REMARKS

I. Preliminary Remarks

The Examiner indicated that the continuing application data listed in the first paragraph of the specification and the declaration are inconsistent. In particular, the declaration omitted the priority claim to U.S. patent application nos. 09/543,562, 09/543,774 and 09/496,914. In order to properly claim priority, the Applicants have submitted herewith an Application Datasheet pursuant to MPEP § 601.05. The application data sheet properly lists the priority claim.

In addition, the Examiner objected to the specification as lacking the status of the continuing applications referred to in the first paragraph of the application. In the foregoing amendment, the status of application nos. 09/543,774 and 09,496,414 is inserted at page 1. The Examiner also indicated that the status of the related applications listed throughout the specification needs to be updated. Accordingly, the status of application no. 09/496,914 at page 12, line 7 was updated in the foregoing amendment.

The specification contains an embedded hyperlink and the Examiner objected to the presence of the hyperlink pursuant to MPEP § 608.01. In the foregoing amendment, the hyperlink was deleted and a description of the web site is now added. The FASTY program is found at the University of Virginia web site and as this is indicated by the hyperlink, the amendment does not add new matter to the specification.

The Examiner objected to the title of the application as allegedly not clearly indicating the invention to which the claims are directed. As suggested by the Examiner, the Applicants replace the original title with the amended title, "METHOD OF PROMOTING STEM CELL PROLIFERATION OR SURVIVAL BY CONTACTING A CELL WITH A NOVEL STEM CELL FACTOR-LIKE POLYPEPTIDE."

The amendments at page 173 and 183 of the specification correct typographical errors. These amendments do not add new matter to the specification.

The amended claims are directed to "ex vivo" methods of promoting stem cell proliferation and maintaining stem cell survival. The term "ex vivo" is supported throughout

the specification including at page 34, lines 3-6, page 44, lines 11-13 and page 91, lines 27-29. This amendment does not add new matter to the application.

II. The rejection under 35 U.S.C. § 112, first paragraph for lack of enablement should be withdrawn.

In paragraph 3, the Examiner rejected claims 62-65 and 74-75 under 35 U.S.C. §112, first paragraph, for assertedly failing to comply with the enablement requirement. However, the Examiner alleges that a large quantity of experimentation is necessary to support that the claimed stem cell growth factors would stimulate stem cell proliferation and differentiation.

The factors to be considered in determining if the amount of experimentation needed is undue are 1) the breath of the claims, 2) the nature of the invention 3) the state of the prior art, 4) the level of one of ordinary skill, 5) the level of predictability in the art, 6) the amount of direction provided by the inventor 7) **the existence of working examples,** and 8) the quantity of experimentation needed to make or use the invention based on the disclosure. *In re Wands*, 858 F.2d 731, 737, 8 USPQ 2d 1400, 1404 (Fed. Circ, 1988). The conclusion that the disclosure is not enabling therefore is not a single factual determination, it must be a conclusion reached by weighing all the factual considerations.

The specification provides working examples to demonstrate that the polypeptides of the present invention stimulate stem cell growth and differentiation. The Examiner **must provide evidence** demonstrating there is reason to doubt the experimental evidence provided in the specification. *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971).

A. Polypeptides that stimulate stem cell growth and differentiation are enabled.

The breath of the claims and the nature of the invention do not require an undue amount of experimentation to meet the enablement requirement because the amended claims are directed to ex vivo methods that use the polypeptides of the invention or variants that are substantially similar to the polypeptides of the present invention. The methods of maintaining ex vivo cultures of stem cells, as well as methods of measuring stem cell proliferation and maintaining stem cell survival are well known in the prior art. Stem cell growth factors were also known in the art at the time of filing; however, the particular

polynucleotide and polypeptide sequences of the present invention were not. Therefore, in light of the state of the prior art, the level of one skill in the art is high. The level of predictability in the art may be low, but the teachings in the specification and the presence of working examples demonstrate that the claims are enabled by the specification. Even though there are eight factors set out in *In re Wands, Id.,* the Examiner bases her rejection for lack of enablement on the lack of guidance provided by the disclosed working examples and the quantity of experimentation needed to enable the claims.

In paragraph 3(i), the Examiner stated that the co-culture growth and differentiation experiments described in Example 6 (page 168, lines 6-15) did not demonstrate that a polypeptide of the present invention induced the increase in mouse stem cell growth. In Example 6, co-cultures were seeded at 20,000 cells ($1x10^4$ stroma cells and $1x\ 10^4$ stem cells) co-cultured in serum-free media. The addition of only IL-3 and IL-6 (vector-transduced stroma cells) to the co-culture caused a 1.5x increase in cell number after 15 days and a 5.25x increase after 18 days. The increase in cell growth was significantly greater in co-cultures containing SCF-1-transfected stroma cells, after 15 days there was a 20.25x increase and after 18 days there was a 41.25x increase. The significant increase in cell number in transfected co-cultures as compared to un-transfected co-cultures demonstrates that SCF-1 is contributing to the increase in cell number. If the addition of IL-3 or IL-6 were the sole factor for causing the increase in cell number, the un-transfected co-cultures would have increased at a similar rate to the transfected co-cultures.

It is known in the art that combinations of growth factors and cytokines support stem cell proliferation. IL-3 and IL-6 are known as supportive factors for *ex vivo* cultures of stem cells (Novelli *et al., Human Gene Therapy* 10: 2927-2940, 1999; Lebkowski *et al., Stem Cells*, 13: 607-612, 1995; Qiu *et al., J. Hematotherapy & Stem Cell Research*, 8: 609-618, 1999). In particular, IL-3 is known to synergize with other growth factors, and is therefore more effective in inducing stem cell growth while in combination with other growth factors (reviewed in Lebkowski *et al., Stem Cells*, 13: 607-612, 1995). Therefore, the addition of IL-3 or IL-6 alone will not cause the maximum increase in cell proliferation.

The Examiner stated that the experiments described in Example 6 are unclear because data for only two time points are disclosed; however, it is irrelevant that cell numbers were not provided for every day the experiment was conducted. The data shown establishes

that there was an increase and there is no reason to doubt the increase in cell number was real. The Examiner also states there was no indication the cells were removed from the media and counted daily. For the experiment to be informative; however, daily counts are not necessary and not a standard in the art. For example, the experiment in Köhler *et al.* (*Stem Cells*, 17:19-24, 1999), measured the effect of feeder layers or growth factors on HUVEC cells after 8 days in culture. Similarly, in Novelli *et al.* (*Human Gene Therapy* 10: 2927-2940, 1999), the effects of culture conditions on hematopoietic blood CD34+ cells were measured after 7 days in culture (see table 1). As the studies in Example 6 were carried out using standard techniques known in the art, the Examiner does not provide any evidence there is a reason to doubt the disclosed experimental data or the techniques standard in the art.

In addition, the Examiner stated the experiments using CD34+ hematopoietic stem cells described in Example 7 (pages 168-170) were unclear. In particular, the Examiner stated that since the experiments were carried out in the presence of cytokines other than SCF-1, it is unclear as to whether SCF-1-induced the stem cell proliferation. The Applicants traverse this statement.

The data in Example 7 demonstrates that in CD34+ stem cell cultures, SCF-1 alone had no effect on cell proliferation but a combination of growth factors induced cell growth. The growth factors used in these studies are commonly added to stem cell supporting media (reviewed in Lebkowski *et al., Stem Cells*, 13: 607-612, 1995; Novelli *et al., Human Gene Therapy* 10: 2927-2940, 1999), but these factors (other than IL-3) also had no effect alone. It is well known in the art that stem cells need a variety of factors for survival, but that does not disparage the fact that when exogenous SCF-1 is included in the culture medium the cell number and morphological changes increase.

The Examiner further stated that the specification provides little guidance for teaching how much growth was or was not observed in the experiments described in Example 7. The effect of exogenous growth factors on cell number was analyzed by viewing the cells under a light microscope after 5 days in culture. The observations were recorded as "(+) indicates growth and/or differentiation; (-) indicates no growth or differentiation and loss of viability of stem cells." One of skill in the art will understand the difference between "loss of viability" and "growth and differentiation." The Examiner has no reason to doubt that an

increase in cell number and a morphological change was observed, and no reason is given why these observations are not believable.

The Examiner again states the experiments in Example 7 only measure proliferation. However, the applicants observed morphological changes which is a characteristic of differentiation and identifying cell lineages. (See Freshney, *Culture of Animal Cells* 2d ed. Wile-Liss, New York, NY 1987 pp 169-170, 187-190). The Applicants also analyzed cell viability (survival) by viewing the cells under the microscope. One method of determined cell viability is determining the net increase in cell number. (See Freshney *Culture of Animal Cells* 2d ed. Wile-Liss, New York, NY 1987 pg. 247). Again, the Examiner has not provided a reason to doubt the veracity of the observations and gives no reason why it is unlikely that SCF-1 in the presence of other cytokines did not induce morphological changes and promote cell growth and cell survival.

The Examiner states that the specification does not provide evidence that the assays were preformed on cells which express the receptor for SCF-1. In these assays, there was an additional increase in cell number (Example 6) and observed cell growth and changes in morphology (Example 7). This data demonstrates that SCF-1 has an effect on stem cells, which is indirect evidence that a receptor for SCF-1 activity is expressed on these cells.

Further, Examples 15-17 (pages 182-186) describe experiments conducted with the murine homolog of SCF-1 (SEQ ID NO: 34). The murine SCF-1 clones was transfected into co-cultured stromal cells (Example 15, pages 182-184) and these transfected stromal cells were co-cultured with murine hematopoietic stem cell (Example 16, pages 184-185). The co-cultures were then transplanted into irradiated mice (Example 17, pages 185-186). As depicted in Figure 7, the co-cultures containing the stromal cells overexpressing murine SCF-1 increased the number of myeloid and lymphoid cells in the peripheral blood of the irradiated mice. As there is high similarity between the mouse and human sequences, this data substantiates the results described in Examples 6 and 7, which demonstrate the human SCF-1 can stimulate stem cell growth and survival. The Examiner failed to consider the experiments carried out with the murine homolog of SCF-1. As stated earlier, the Examiner must consider **all experimental evidence** as a whole when evaluating if the specification enables the invention. At the time of filing, one of skill in the art would consider the assays described in Examples 6, 7 and 17 to be routine. There is much art describing the cytokines

(such as IL-3, IL-6, Flt, KIT) needed to support a stem cell culture (reviewed in Lebkowski *et al., Stem Cells*, 13: 607-612, 1995) and one of skill in the art will clearly understand the addition of these factors support stem cell growth; however, the addition of SCF-1 of the present invention caused cell number to increase beyond the support of the known factors. Therefore, the level of skill in the art at the time of filing would understand how to make and use the invention and would characterize SCF-1 as a factor that stimulates stem cell growth and survival.

For a proper enablement rejection, the Examiner must consider the factors set out in *In re Wands, Id.* and not limit her reasoning to one or two factors. In considering all these factors, the specification enables ex vivo methods of stimulating stem cell proliferation or maintaining stem cell survival. In paragraph 3 of the Action (page 8), the Examiner encouraged the Applicants to provide further experimental evidence to support the specification. Even though further data is not included herein, the specification, including the working examples, enables the claims, and the Examiner must provide evidence demonstrating there is reason to doubt the working examples.

B. In vivo methods of promoting stem cell proliferation and survival are enabled.

In paragraph 3(ii), the Examiner alleged the specification does not support proliferation or survival of a stem cells *in vivo*. The Applicants traverse this statement. The experiments in Example 17 (as described above) demonstrate murine SCF-1 supports stem cell proliferation *in vivo* in irradiated mice. However, in order to expedite prosecution, the claims have been amended to be directed to *ex vivo* methods. The Applicants reserve the right to prosecute *in vivo* methods in a continuing application.

C. Polypeptides that are 85% identical to SEQ ID NO: 13, 32 or 34 are enabled.

In paragraph 3(iii), the Examiner alleged that polypeptides that are 85% identical to SEQ ID NO: 13 are not enabled by the specification. The Applicants traverse this rejection.

Example 1 teaches how to identify polynucleotides that encode polypeptides that are 85% identical to SEQ ID NO: 13 using EST probes to screen cDNA libraries. (See specification page 54 line 13 through page 55, line 18 and pages 164-166) and to ultimately

clone the polynucleotide sequence. (See specification page 168). Therefore, the starting materials are taught in the specification and the methods to identify and make the variant sequences, such as synthesizing polynucleotides and amplification of native polynucleotides, are conventional in the art. In addition, the specification provides multiple commercially available computer programs which will assess nucleotide sequence similarity to SEQ ID NO: 13, 32 or 34 such as GAP, BLASTP and FASTA. (See specification pages 77, lines 5-25) The specification also guides one of skill in the art to determine which nucleotides can be altered within a polynucleotide sequence while the encoded polypeptide retains the required biological activity. Assays for measuring stem cell growth factor activity are described at page 91, line 23 through page 96, line 5. In addition, assays for determining cytokine production and cell proliferation, survival and differentiation are described at page 89, line 29 through page 92, line 20.

The specification teaches how to make (identify, isolate and clone) and use three SCF-1 polynucleotide sequences that are greater than 85% identical to each other. Identification and cloning of the polynucleotide sequence of SEQ ID NO: 12 which encodes the amino acid sequence of SEQ ID NO: 13 is described in Examples 1-5 (pages 164-168). The polynucleotide sequence of SEQ ID NO: 33 is 98.5% identical to SEQ ID NO: 12 and encodes an amino acid sequence (SEQ ID NO: 34) that is 100% identical to SEQ ID NO: 13. The murine polynucleotide sequence of SEQ ID NO: 31 is 88.5% identical to SEQ ID NO: 12 and encodes an amino acid sequence (SEQ ID NO: 33) that is 87.1% identical to SEQ ID NO: 13. The identification and cloning of SEQ ID NOS: 32 and 33 is described in Example 13 (pages 180-181).

As described above, the specification provides working examples that demonstrate the polypeptide of SEQ ID NO: 13, 32 or 34 increased stem cell number and maintained stem cell survival in culture assays described in Examples 6 and 7. (See pages 167-170). In addition, the polypeptide of the invention also induced morphological changes in stem cells in these experiments. (See charts on pages 169 and 170). These experiments demonstrate that the polypeptides of the present invention increase stem cell growth, survival and differentiation.

D. Conclusion

In light of the foregoing amendment and remarks, the Applicants request the rejection of claims 62-65 and claims 74-75 under 35. U.S.C. § 112, first paragraph for lack of enablement be withdrawn.

III. The rejection under 35 U.S.C. § 112, first paragraph, for asserted lack of adequate written description should be withdrawn.

In paragraph 4, the Examiner rejected claim 62 under 35 U.S.C. § 112, first paragraph, for assertedly failing to satisfy the written description requirement. In particular, the Examiner alleged that the specification does not teach functional or structural characteristics of all possible derivatives of SEQ ID NO: 13, 32 and 34 and in particular polypeptides with at least 85% sequence identity to the amino acid sequence of SEQ ID NO: 13, 32 or 34.

Amended claim 62 is directed to a method of promoting stem cell or germ cell proliferation comprising contacting said cell with a polypeptide which comprises an amino acid sequence that is at least 85% identical to SEQ ID NO: 13, 32 or 34 and exhibits stem cell growth factor activity. Similarly, new claim 76 is directed to a method of maintaining stem cell or germ cell survival comprising contacting said cell with a polypeptide which comprises an amino acid sequence that is at least 85% identical to SEQ ID NO: 13, 32 or 34 and exhibits stem cell growth factor activity. The polypeptides recited in these claims are structurally and functionally defined and therefore these claims meet the Written Description Guidelines of the United States Patent and Trademark Office (February, 2000). In particular, Example 14 of the Written Description Guidelines (pp. 53-55) teaches that a claimed variant polypeptide that is substantially similar to a sequence taught in the specification, along with a functional limitation that the claimed variant polypeptides exhibit a specified catalytic activity, meets the written description requirement if the required activity can be determined as described in the specification.

In the instant case, the claimed variants must be at least 85% identical to SEQ ID NO: 13 and therefore are substantially similar to the sequences taught in the specification. In addition, the claimed variants are limited to those that exhibit stem cell growth factor activity. The activities encompassed by the term "stem cell growth factor activity" are set out in the specification at page 91, line 23, through page 92, line 7. As described above, the

specification teaches assays to measure the stem cell growth factor activity at page 94, lines 10-18, and page 95, line 12, through page 96, line 5, and provides working examples demonstrating that the disclosed polypeptides increase stem cell growth, survival and differentiation. Due to the substantial sequence similarity to SEQ ID NO: 13, 32 or 34 and clearly defined functional limitations, the polypeptides recited in claims 62 and 76 are adequately described in the specification.

IV. The rejection under 35 U.S.C. § 112, second paragraph, should be withdrawn.

In paragraph 7 of the Action, the Examiner rejected claims 62-65 and 74-75 as allegedly begin indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. In particular, the Examiner asserted that the term "supporting" is not defined by the claim or the specification.

Amended claim 62 is directed to a method of promoting stem and germ cell proliferation, while new claim 76 is directed to a method of maintaining stem and germ cell survival. Thus, the claims do not recite the term "supporting" and the rejection under 35 U.S.C. § 112, second paragraph is now moot. The Applicants request this rejection be withdrawn.

CONCLUSION

In view of the foregoing amendment and remarks, claims 62-65 and 74-77 are in condition for allowance and the applicants request notification of the same.

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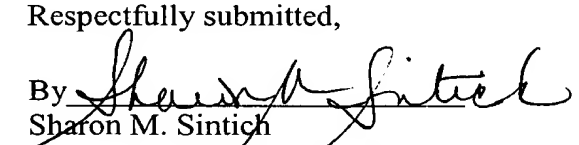
Respectfully submitted,

By 

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Ex Vivo Expansion of CD34$^+$ Umbilical Cord Blood Cells in a Defined Serum-Free Medium (QBSF-60) with Early Effect Cytokines

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ABSTRACT

To investigate the clinically applicable conditions that support substantial expansion of both primitive and more mature hematopoietic cells of umbilical cord blood (UCB) for transplantation in adults, enriched CD34$^+$ cells from 8 fresh UCB samples and 4 expanded UCB products were cultured in defined serum-free medium (QBSF-60) in the presence of a cytokine combination of SCF, Flt-3-ligand (FL), thrombopoietin (TPO), IL-3 for up to 2 weeks. Fresh medium with cytokines was supplemented or exchanged at day 4, day 7, and day 10. The proliferative response was assessed at day 7, day 10, and day 14 by evaluating the following parameters: nucleated cell (NC), clonogenic progenitors (colony-forming unit–granulocyte-macrophage [CFU-GM], burst-forming unit-erythrocyte [BFU-E], CFU-GEMM, and high-proliferative potential colony-forming cell [HPP-CFC]), immunophenotypes (CD34$^+$ cells and CD34$^+$ subpopulations), and LTCIC. Simultaneously numerical expansion of various stem/progenitor cells, including primitive CD34$^+$CD38$^-$HLA-DR$^-$ subpopulation and LTCIC, CD34$^+$ cells, and clonogenic progenitors to mature nucleated cells, were continuously observed during the culture. An average $103.32 \pm 71.37 \times 10^6$ CD34$^+$ cells (range 10.12×10^6–317.9×10^6) could be obtained from initial $1.72 \pm 1.13 \times 10^6$ UCB CD34$^+$ cells after 10–14 days cultured under the described conditions. Sufficient CD34$^+$ cells ($>50.0 \times 10^6$) for transplantation in adults would be available in all but one UCB collections after 10–14 days expansion. The expanded CD34$^+$ cells sustained most of the in vitro characteristics of initial unmanipulated CD34$^+$ cells, including clonogenic efficiency (of both primitive and committed progenitors), the proportion of CD34$^+$CD38$^-$HLA-DR$^-$ subpopulation, and the expansion potential. Initial addition of IL-3 to the cocktail of SCF + FL + TPO had positive effects on the expansion of both primitive and, especially, the more mature hematopoietic cells. It accelerated the expansion speed and shortened the optimal culture time from 14 days to 10 days. These results indicated that our proposed short-term culture system, consisting of QBSF-60 serum-free medium with a simple early acting cytokine combination of SCF + FL + TPO, could substantially support simultaneous expansion of various stem/progenitor cell populations involved in the different phases of engraftment. It would be a clinically applicable protocol for ex vivo expansion of CD34$^+$ UCB cells.

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INTRODUCTION

UMBILICAL CORD BLOOD (UCB) HAS BEEN USED as a source of hematopoietic stem cells (HSC) for transplantation and is proving to be an acceptable alternative to bone marrow (BM) (1–6). Over the past 9 years, more than 1200 transplants using related and unrelated UCB have been performed, with promising results (5). The use of UCB for stem cell transplantation has many advantages, including widespread availability and potentially reduced GvHD (2–5). However, the limitations for the more widespread application of UCB are emerging as experience accumulates in the clinical setting. The major limitation is the low number of cells available from most UCB collections. This low cell number may contribute to delayed engraftment of neutrophils and platelets and may limit the general use of UCB in adults and larger pediatric patients (2–5).

In view of the potential limitation of the low cell number available in a UCB collection, many investigators have studied ex vivo expansion of UCB hematopoietic cells to obtain the quantity and quality of UCB cells required for successful (i.e., rapid, complete, and sustained) long-term hematopoietic reconstitution after transplantation in adults (7–12). Although many protocols for ex vivo expansion of UCB cells have been described, in few reports have all stem/progenitor cell compartments (especially $CD34^+$ cell, $CD34^+$ subpopulations, and LTCIC) been studied to determine whether expanded UCB cells still contain long-term repopulating cells, which is of critical relevance for clinical application (7–12). Some studies questioned if sufficient $CD34^+$ cells for transplantation in adults could be obtained from a UCB collection after expansion (10–12). In our previous study (13), we found that $>50 \times 10^6$ $CD34^+$ cells, which would be sufficient for transplantation in adults, could be produced in about 30% of UCB collections after 7 days expansion in 20% FBS-IMDM medium in the presence of SCF plus Flt3-ligand (FL) associated with a 136GE (IL-1β + IL-3 + Il-6 + G-CSF + erythropoietin [EPO]) cocktail. Recently, Piacibello et al. (14) described a serum-containing long-term expansion system that allows a substantial amplification of UCB primitive hematopoietic progenitors, including $CD34^+/CD38^-$ cells and LTCIC after 20 weeks in the presence of a simple two cytokine combination of FL and thrombopoietin (TPO). However, for a clinically relevant expansion, serum is undesirable, and a shorter culture period would be preferable.

In the present study, we describe a clinically applicable short-term expansion system, which consists of a defined serum-free medium QBSF-60 and an early acting cytokine combination of SCF + FL + TPO with or without IL-3, that supported substantial expansion of both primitive and more mature hematopoietic cells.

MATERIALS AND METHODS

Cell preparation

Human UCB samples were collected from healthy full-term neonates immediately after delivery by gravity into a 450-ml blood donor set containing 20 ml citrate-phosphate-adenine-1 (CPD-A) anticoagulant (Baxter Health Care, Deerfield, IL) as described previously by Rubinstein et al. (15). Nucleated cells (NC) were separated by sedimentation with 1.2% (final, wt/vol) hydroxyethyl starch in 0.9% sodium chloride (HES) (Hespan, DuPont, Wilmington, DE), followed by RBC lysis with ammonium chloride (NH_4Cl) hypotonic solution.

Purification of UCB $CD34^+$ cells

The $CD34^+$ cells were isolated with the miniMACS immunomagnetic separation device by using a CD34 isolation kit or CD34 multisort kit (Miltenyi Biotech Inc., Auburn, CA) according to the instructions of the manufacturer. To improve the purity, cells in the $CD34^+$ fraction were applied to a second column, and the purification steps were repeated.

Expansion culture

On day 0, enriched $CD34^+$ UCB cells (2.0×10^4/ml) were cultured in a 25-cm^2 vented flask in 5–10 ml defined serum-free medium QBSF-60 (kindly provided by Quality Biological Inc., Gaithersburg, MD) containing 50 ng/ml SCF (Sigma, St. Louis, MO), 100 ng/ml FL (kindly provided by Immunex Co., Seattle, WA), and 100 ng/ml TPO (kindly provided by Genentech Inc., San Francisco, CA) with or without 20 ng/ml IL-3 (Sigma). On day 5, equal volumes (5–10 ml) of fresh medium with the same doses of FL and TPO (100 ng/ml) and half-doses of SCF (25 ng/ml) were added. On day 7 and day 10, half (5 ml) of the medium and cells from each flask were harvested for tests, then equal volumes (5 ml) of fresh medium with 100 ng/ml FL and 100 ng/ml TPO were added. On day 14, all cells were harvested for tests and reselection of $CD34^+$ cells (4 cases). At each time point, the proliferative response was assessed by evaluating the following parameters: total NC cells, clonogenic progenitors (colony-forming unit–granulocyte-macrophage [CFU-GM], burst-forming unit-erythrocyte [BFU-E], CFU-GEMM, and high-proliferation potential colony-forming cell [HPP-CFC]), immunophenotypes ($CD34^+$ cells and $CD34^+$ subpopulations), and LTCIC.

Progenitor colony-forming assay

The assay for CFU-GM, BFU-E, CFU-GEMM, and HPP-CFC was performed in 35-mm Petri dishes in 1-mL

aliquots of 0.9% methylcellulose in IMDM containing 30% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol, and 2 mM L-glutamine, with recombinant cytokines: 50 ng/ml SCF, 20 ng/ml IL-3, 20 ng/ml IL-6, 20 ng/ml G-CSF, 20 ng/ml GM-CSF, and EP IU/ml 3 (MethoCult™ CF+ H4435) (StemCell Technologies, Vancouver, BC, Canada). Cells were incubated at 37°C in 5% CO_2 in a humidified atmosphere for 14–28 days. Three dishes were set up for each individual data point per experiment. CFU-GM, BFU-E, and CFU-GEMM colonies were enumerated on days 14–21, and HPP-CFC colonies (dense colony with diameters >0.5 mm, cells > 50,000) were scored on day 28 (16).

LTCIC assays

The LTCIC assay was performed on the feeder layer of irradiated (6000 cGy) murine M2-10B4 cells (3.0×10^4/cm^2)(ATCC 1972-CRL, ATCC, Rockville, MD). Briefly, test cell suspensions (2×10^5/ml/well of fresh NC or expanded cells or 1.0×10^4/ml/well of CD34+ fraction cells) were seeded on a prepared M2-10B4 feeder layer in 24-well plates in modified Dexter's long-term BM culture (LTBMC) medium consisting of IMDM (Sigma) with 12.5% horse serum (HS) (StemCell Technologies), 12.5% FBS (StemCell Technologies), 10^{-4} M 2-mercaptoethanol (Sigma), 10^{-6} M hydrocortisone (Sigma), and 2 mM L-glutamine (Sigma). Four to eight wells were set up for each individual data point per experiment. Cultures were demidepopulated weekly by removal of half of the culture volume, followed by replacement with fresh medium. After 5 weeks in culture, nonadherent and adherent cells harvested by trypsinization were pooled, washed, and assayed together for clonogenic cells in standard methycellulose cultures as described.

Flow cytometry

CD34+ cells and CD34+ subpopulations were analyzed using three-color flow cytometry. Fresh UCB NC or expanded cells (1×10^6) and 2×10^5 CD34+ fraction cells were labeled with FITC-conjugated anti-CD34 (clone HPCA-2) (Becton Dickinson, San Jose, CA), PE-conjugated anti-CD38 (clone HIT2) (PharMingen, San Diego, CA); cy-chrome (Cy)-conjugated anti-HLA-DR (clone G46-6) (PharMingen), PE-conjugated CD7 (clone M-T701) (PharMingen), and Cy-conjugated CD19 (clone B43) (PharMingen). Two control samples were used. Cells in the first control were labeled with isotype control for FITC. The second control was labeled with FITC-conjugated anti-CD34 and isotype controls for PE and Cy. Three-color antibody marker analysis was performed on a FACScan flow cytometry configured with Lysis II software (Becton Dickinson).

Statistical analysis

All results were expressed as the mean ± SD of data from eight separate experiments except those indicated. A paired Student's *t*-test was used for statistical analysis.

RESULTS

Cellular yields in initial UCB samples

Twenty-four fresh UCB collections had been processed using our UCB cell processing protocol. A single UCB collection had an average 73.5 ± 25.9 ml (range 35–150 ml) volume, with $9.1 \pm 3.8 \times 10^6$ WBC/ml (range $2.4–18.6 \times 10^6$). The percent of CD34+ cells present among the NC from fresh UCB was 0.51% ± 0.16% (range 0.15%–0.81%). The yields of NC and CD34+ cells were 79.4% ± 6.7% (range of 64.8%–92.3%) and 70.8% ± 8.8% (range 53.6%–91.1%), respectively. After cell processing, an average $4.95 \pm 3.17 \times 10^8$ NC (range $2.19–13.71 \times 10^8$) and $2.09 \pm 1.34 \times 10^6$ CD34+ cells ($n = 20$, range $0.51–6.88 \times 10^6$) would be obtained from a single UCB sample.

Expansion efficiency

The expansion efficiency is expressed by the fold expansion of an evaluated item at each time point during the culture with respect to the input cells. The results on day 7, day 10, and day 14 are detailed in Table 1. Simultaneously, numerical expansion of various stages of UCB hematopoietic cells, including the very primitive CD34+CD38−HLA-DR− subpopulation and LTCIC CD34+ cell population, clonogenic progenitors to mature NC was continuously observed during the culture in both groups with or without initial addition of IL-3. On day 7 and day 10, the differences between the two groups were statistically significant in all evaluated items ($p < 0.05$). On day 14, only the fold expansions of NC and CFU-C in the group with IL-3 were significantly higher than those in the group without IL-3 ($p < 0.05$). However, there were no significant differences observed in all evaluated items when the data in the group without IL-3 on day 14 was compared with those in the group with IL-3 on day 10 ($p > 0.05$).

Total available CD34+ cells after expansion

To determine if sufficient CD34+ cells for transplantation in adults would be obtained from a single UCB collection, we calculated the total available CD34+ cells in each UCB sample based on all selected CD34+ cells from

TABLE 1. FOLD EXPANSION OF HPC[a]

Day	Group	Nucleated cells	$CD34^+$ cells	$CD34^+CD38^-DR^-$ cells	CFU-C	HPP-CFC	LTC-IC[b]
7	−IL-3[c]	74.6 ± 22.3	20.9 ± 6.3	5.5 ± 1.9	22.5 ± 4.1	23.4 ± 6.4	6.8 ± 2.8
	+IL-3[d]	157.0 ± 38.0	31.1 ± 8.3	11.5 ± 3.6	36.6 ± 7.3	36.8 ± 7.7	13.6 ± 5.0
10	−IL-3	236.0 ± 42.0	39.5 ± 10.2	23.2 ± 4.8	42.8 ± 10.5	46.2 ± 13.8	14.8 ± 5.4
	+IL-3	532.0 ± 62.3	55.8 ± 15.8	44.7 ± 10.1	69.9 ± 12.6	60.0 ± 13.4	26.2 ± 7.2
14	−IL-3	590.0 ± 58.0	65.5 ± 11.8	49.3 ± 9.8	67.3 ± 13.8	65.2 ± 12.6	30.6 ± 8.0
	+IL-3	876.0 ± 64.8	73.4 ± 19.3	58.4 ± 11.6	90.9 ± 18.6	81.6 ± 14.0	36.8 ± 11.4

[a]Results are mean ± SD fold increase of the input values (values at day 0) of 8 samples (except [b]$n = 4$).
[c]Without addition of IL-2 at initial culture.
[d]With addition of IL-3 at initial culture.

fresh UCB collections cultured for expansion. An average 103.32 ± 71.37 × 10^6 $CD34^+$ cells (range 10.12 × 10^6–317.9 × 10^6) could be obtained from an initial 1.72 ± 1.13 × 10^6 UCB $CD34^+$ cells after 10–14 days of culture. Table 2 shows the data of each tested UCB sample at each evaluated time point during the expansion culture. Initial addition of IL-3 caused much higher expansion of $CD34^+$ cells on day 7 and day 10. On day 14, the expansion of $CD34^+$ cells in the two groups was very close. There was no statistical difference observed when comparing the results of expansion without IL-3 on day 14 with expansion on day 10 when IL-3 was included in the cytokine combination ($p > 0.05$).

Dynamic changes in $CD34^+$ cells and $CD34^+$ subpopulations

The immunophenotypic kinetics were analyzed by three-color flow cytometry. $CD34^+$ cells and $CD34^+$ subpopulations with coexpressions of CD38/HLA-DR and CD7/CD19 molecules were evaluated. The results are shown in Figure 1. The percentage of $CD34^+$ cells quickly dropped in the first week and decreased further but more slowly in the second week. On day 14 of expansion, the $CD34^+$ cells represented 6.35% and 4.92% of total cell populations, respectively, in the group without IL-3 and the group with IL-3, which represented a 1 log increase in $CD34^+$ cells over the initial fresh UCB NC. Of the $CD34^+$ cell populations, the very primitive $CD38^-$HLA-DR^- subpopulation dropped to the lowest level on day 7 and then quickly recovered to close to the initial level on day 14. The proportions of primitive $CD38^-$HLA-DR^+ and $CD38^+$HLA-DR^- subpopulations were increased to >2-fold of the initial levels on day 10. Then both decreased slightly on day 14 but were still significantly higher than the initial levels. The initial addition of IL-3 resulted in a higher proportion of the $CD38^+$HLA-DR^- subset and a lower proportion of the

TABLE 2. TOTAL $CD34^+$ CELLS AVAILABLE AFTER EXPANSION[a]

		Day 7		Day 10		Day 14	
UCB no.	Day 0	−IL-3[b]	+IL-3[c]	−IL-3	+IL-3	−IL-3	+IL-3
011	1.43	48.82	54.81	73.36	81.51	89.38	111.83
012	1.81	43.82	62.70	76.71	128.33	141.61	148.96
013	2.16	39.51	48.86	73.03	91.22	147.61	160.40
014	1.14	25.54	36.94	44.41	62.24	74.08	78.20
015	1.34	44.41	33.86	69.75	75.21	97.77	72.94
016	0.51	6.89	10.62	10.12	15.97	20.11	22.93
017	1.13	23.57	50.73	45.10	86.49	89.64	123.80
018	4.25	75.86	126.91	155.21	254.83	274.38	317.96
Average −IL-3	1.72 ± 1.13	35.78 ± 21.14		68.48 ± 41.69		115.29 ± 74.49	
+IL-3	1.72 ± 1.13	51.38 ± 33.81		99.89 ± 71.59		129.63 ± 88.1	

[a]All values ×10^6 are calculated based on all enriched $CD34^+$ cells cultured for expansion and fold expansion at each time point in each experiment.
[b]Without addition of IL-2 at initial culture.
[c]With addition of IL-3 at initial culture.

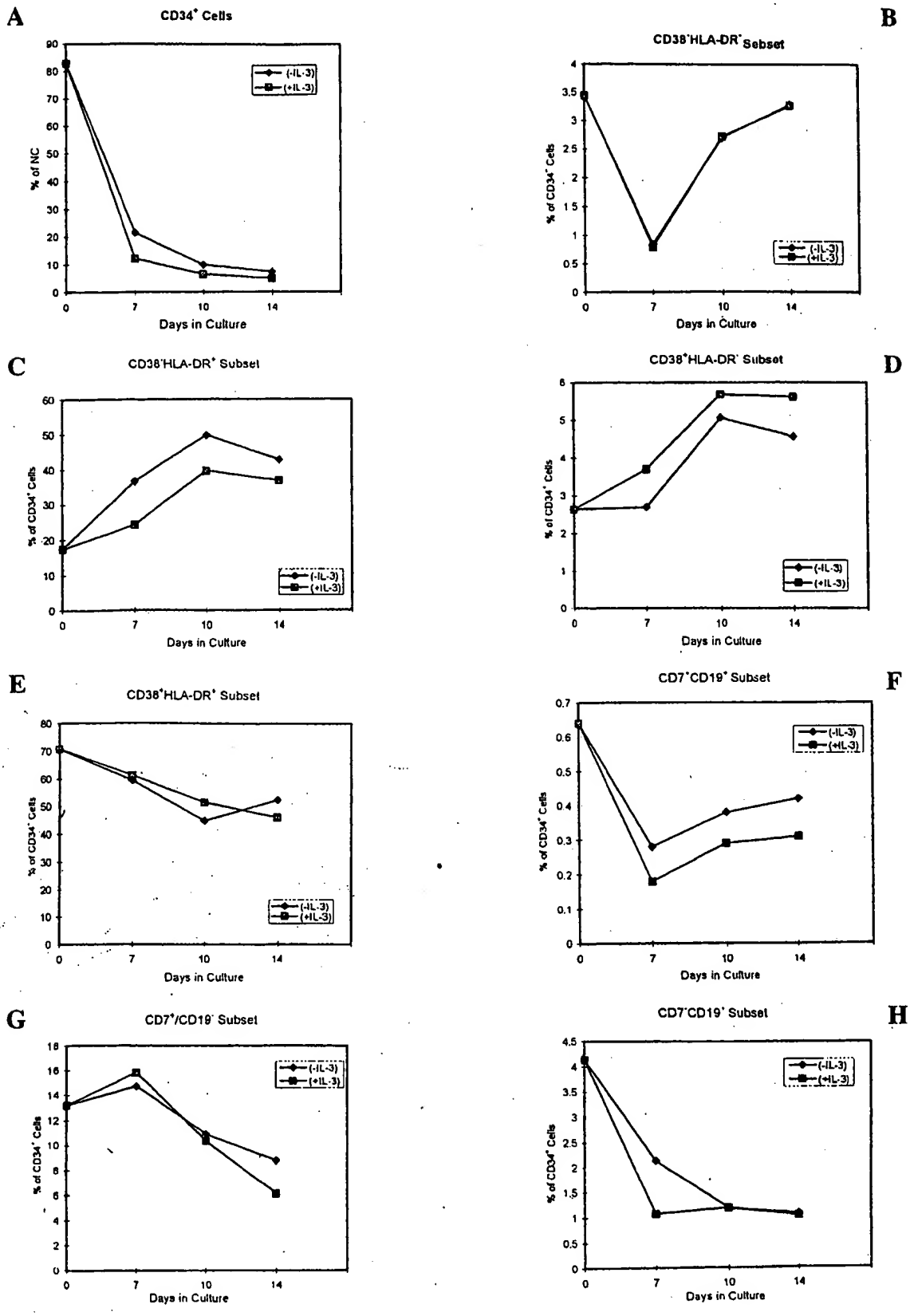

FIG. 1. Kinetics of CD34+ cells and CD34+ subpopulations during expansion culture. Enriched CD34+ UCB cells were cultured in QBSF-60 serum-free medium in the presence of FL + SCF + TPO with or without addition of IL-3 at initial culture for 2 weeks. Immunophenotypes were analyzed using three-color flow cytometry at each tested time point. All values are the median of eight independent experiments. + IL-3, with addition of IL-3 at initial culture; − IL-3, without addition of IL-3 at initial culture.

$CD38^-HLA\text{-}DR^+$ subpopulation. The proportion of more mature $CD38^+HLA\text{-}DR^+$ continuously and slowly decreased in both groups during culture. The proportions of all three subpopulation of $CD34^+$ lymphocytic precursors were significantly decreased compared with their initial levels. The $CD7^+CD19^+$ subset quickly dropped to a very low level in the first week and then steadily recovered to about half the initial level. The initial addition of IL-3 resulted in a lower proportion of the $CD7^+CD19^+$ subset. The $CD7^+CD19^-$ subset increased slightly in the first week and then dropped continuously to half (without IL-3) and one third (with IL-3) of the initial level, respectively. The $CD7^-CD19^+$ subset decreased from an initial 4.1% to about 1% on day 7 (with IL-3) or day 10 (without IL-3), then maintained this level.

Comparison of in vitro characteristics of selected $CD34^+$ cells

To further evaluate the in vitro characteristics of the expanded UCB HCP, we studied reselection of $CD34^+$ cells from 4 expanded UCB products on day 14 of expansion. The clonogenic efficiency, $CD34^+$ subpopulations, and expansion potential of the expanded $CD34^+$ cells were compared with the initial $CD34^+$ UCB cells. The clonogenic efficiency, including the yield of primitive LTCIC, HPP-CFC, and committed CFU-C from expanded $CD34^+$ cells, was comparable to the yield from the initial $CD34^+$ cells (Table 3). Compared with the subpopulations in the initial $CD34^+$ cells (Table 4), the very primitive $CD38^-HLA\text{-}DR^-$ subpopulation remained unchanged in expanded $CD34^+$ cells. The $CD38^-HLA\text{-}DR^+$ and $CD38^+HLA\text{-}DR^-$ subpopulations in expanded $CD34^+$ cells increased 101.8% and 82.5%, respectively, over those in the initial $CD34^+$ cells, whereas the more mature $CD38^+HLA\text{-}DR^+$ subpopulation decreased 25.9%. The proportions of all three subpopulations of $CD34^+$ lymphocyte precursors were significantly decreased in expanded $CD34^+$ cells. Compared with the initial $CD34^+$ cells, the expansion potential (expressed by fold expansion in evaluated items) of the expanded $CD34^+$ cells was not significantly changed, although there was a trend toward a slight decrease in the six evaluated items (Table 5).

TABLE 3. COMPARISON OF CLONOGENIC EFFICIENCY OF ENRICHED $CD34^+$ CELLS[a]

$CD34^+$ cells	*CFU-C*	*HPP-CFC*	*LTC-IC*
Initial ($n = 8$)	24.8 ± 4.6	0.92 ± 0.30	0.42 ± 0.22[b]
Expanded ($n = 4$)	21.5 ± 2.8	0.88 ± 0.20	0.38 ± 0.16
p value	>0.05	>0.05	>0.05

[a] Initial $CD34^+$ cells were selected from 8 fresh UCB collections. Expanded $CD34^+$ cells were selected from expanded UCB products on day 14 of expansion in the presence of SCF + FL + TPO, with initial addition of IL-3.
[b] $n = 4$.

DISCUSSION

Because of the relatively low cell numbers available in a UCB collection, the first question about the general use of UCB for allogeneic hematopoietic stem cell transplantation (HSCT) has been about the engraftment potential of a single UCB unit in adult patients (3–6). Early estimates suggested that there may be enough hematopoietic stem/progenitor cells in an average UCB sample to reconstitute adult patients, but the ability to engraft an adult may require growth factor mediated ex vivo expansion (7). Therefore, there is great interest in the investigation of ex vivo UCB HPC to obtain the quantity and quality of UCB cells for successful transplantation (i.e., rapid, complete, and sustained long-term hematopoietic reconstitution) in adult and in larger pediatric patients (7–14). Although many protocols for ex vivo expansion of UCB cells have been described, a clinically applicable expansion culture system that defines the optimal cytokine combination, media, and time for the simultaneous and substantial expansion of the various stem/progenitor cell populations involved in the different phases of engraftment is still to be established (10–12).

UCB-derived $CD34^+$ cells possess a much higher ex vivo proliferation and expansion potential than do adult BM-derived $CD34^+$ cells (6,17–21). The ability of HPC to express an intrinsic expansion and proliferation potential in vitro will depend on the cytokines present in culture (6,22–24). A number of previous reports have

TABLE 4. COMPARISON OF $CD34^+$ SUBPOPULATIONS IN ENRICHED $CD34^+$ CELLS[a]

	$CD38^-DR^-$	*$CD38^-DR^+$*	*$CD38^+DR^-$*	*$CD38^+DR^+$*	*$CD7^+CD19^+$*	*$CD7^+CD19^-$*	*$CD7^-CD19^+$*
Initial	3.44	17.44	2.63	76.49	0.64	13.17	4.13
Expanded	3.30	35.20	4.80	56.70	0.34	6.81	1.60

[a] All values are median % of $CD34^+$ cells, analyzed by three-color flow cytometry. Initial $CD34^+$ cells were isolated from fresh UCB samples ($n = 8$). Expanded $CD34^+$ cells were isolated from expanded UCB products on day 14 of expansion in the presence of SCF + FL + TPO with initial addition of IL-3 ($n = 4$).

TABLE 5. COMPARISON OF EXPANSION POTENTIAL OF ENRICHED CD34+ CELLS[a]

	NC	CD34+ cells	CD34+CD38-DR-	CFU-C	HPP-CFC	LTC-IC
Initial (n = 8)	876.0 ± 64.8	73.4 ± 19.3	58.4 ± 11.6	90.9 ± 18.6	81.6 ± 14.0	36.8 ± 11.4
Expanded (n = 4)	798.0 ± 76.6	69.8 ± 21.1	49.6 ± 13.5	85.8 ± 20.9	78.2 ± 12.6	28.2 ± 9.8

[a] All results are mean ± SD fold increase of input values (values at day 0) on day 14 in QBSF-60 serum-free medium in the presence of SCF + FL + TPO with initial addition of IL-3. Initial CD34+ cells were isolated from fresh UCB samples. Expanded CD34+ cells were isolated from expanded UCB products on day 14 of expansion in the presence of SCF + FL + TPO with initial addition of IL-3.

studied the effects of cytokine combinations on UCB-derived CD34+ cell expansion. Assessed cytokines (either alone or in combination) include SCF, IL-1, IL-3, IL-6, IL-11, GM-CSF, G-CSF, M-CSF, EPO, and in a few reports, TPO and FL (7–14,22–26). The best results have been obtained when cytokines were used in combinations that included early acting cytokines, such as SCF, FL, and TPO (10,13,14,22–24). SCF is a key regulator of hematopoiesis and is the most used cytokine in ex vivo expansion of human HPC (7–13,22–24). Both FL and TPO are newly cloned cytokines. Several studies have indicated that FL and TPO may play key rules in early human hematopoiesis through their ability to potently and synergistically promote the growth of multipotent stem/progenitor cells in combination with other early acting cytokines, such IL-3, IL-6, IL-11, and in particular with SCF (27–35). Synergistic effects have been observed in maintenance and expansion of primitive stem/progenitor cells during ex vivo expansion when any two of the three cytokines, SCF, FL, and TPO, were combined in a cytokine cocktail (10,13–14,22–24,33–35). Our data clearly demonstrate that the combination of these three cytokines could efficiently support simultaneous expansion of various stages of UCB HPC, from very primitive CD34+CD38-HLA-DR- subpopulation and LTCIC, CD34+ cells, and HPP-CFC to more mature committed progenitors (CFU-C), without exhausting the proliferative potential in the UCB-derived CD34+ cell expansion.

IL-3 has been considered essential for effective expansion of human HPC (22–24). However, it has been reported that IL-3 may suppress the earliest process of hematopoiesis and may negatively affect the ability of cultured cells to engraft the marrow of recipient mice (36–38). Because of concern about this issue, IL-3 was added only in the initial culture and was not included in subsequent media/cytokine supplements on day 5, day 7, and day 10. We also evaluated the impact of initial addition of IL-3 on the expansion potential of CD34+ UCB cells and the in vitro characteristics of expanded CD34+ cells in the presence of SCF, FL, and TPO. Our data show that the initial addition of IL-3 to the early acting cytokine cocktail of SCF + FL + TPO markedly augmented the committed progenitors and mature NC and slightly increased the output of primitive CD34+CD38-HLA-DR- subpopulation and LTC-IC, CD34+ cells and HPP-CFC. The initial addition of IL-3 accelerated the expansion speed and shortened the optimal culture period from 14 days to 10 days. These results were in agreement with some other reports on ex vivo expansion of human CD34+ hematopoietic cells (10,28,32). Moreover, our more recent results indicate that the initial addition of IL-3 is necessary for more efficient expansion of UCB-derived CD34+ cells from frozen UCB samples.

Traditionally, in vitro hematopoietic cell culture assays were performed in media containing relatively high concentrations of animal sera, such as FBS. The composition of the serum is poorly defined and variable because of source variations. Media with better defined compositions are, therefore being employed increasingly to study hematopoiesis (39). As serum is undesirable for clinical application because of concerns about allergens associated with nonhuman proteins and possible viral contamination, a number of defined serum-free media have been developed over the last few years to support hematopoietic cell cultures (40–42). QBSF-60 is a defined serum-free medium originally designed to support the ex vivo expansion of CD34+ progenitor cells by Quality Biological, Inc. Our results indicate that QBSF-60 could effectively support substantial expansion of UCB-derived CD34+ cells.

It is not known how few hematopoietic stem/progenitor cells are needed to repopulate the blood cell system of an adult. When collections of autologous or allogeneic BM cells are made for such purpose, it is usual to collect at least 2–4 × 10^8 NC/kg. From published reports, it is interesting to note that the number of UCB cells infused is 1 log fewer than that given for BM or for peripheral blood stem cell transplantation (PBSCT) (1–5). Although this finding indirectly supports the concept that UCB hematopoietic cells possess higher proliferative and repopulating potential (6), most UCB transplant reports have demonstrated that a low cell number infused was associated with both an increased risk of nonengraftment and a delay of engraftment (2–5). Glukman et al (5) suggest that the minimum cell number infused for UCB

transplant should be $>2 \times 10^7$ NC/kg, and 3.7×10^7 NC/kg infused is a good prognostic factor for engraftment and survival. Even assuming that 2×10^7 NC/kg is the threshold for engraftment when UCB is used, there are <25% of UCB units containing sufficient NC to engraft patients weighing over 50 kg (5,10,15,43,44). $CD34^+$ cells present most of the mature and immature hematopoietic progenitors, including pluripotent stem cells (45,46). Enriched $CD34^+$ cells from peripheral blood have successfully reconstituted hematopoiesis after autologous and allogeneic transplantation (47,48). The number of $CD34^+$ cells infused is a good indicator for engraftment; $1–2 \times 10^6$ $CD34^+$ cells/kg has been widely used as the threshold door in allogeneic transplantation (46). However, $CD34^+$ cell enumeration has not been used as an engraftment indicator in UCB transplant because of the difficulties in quantification of $CD34^+$ cells in UCB (4,5). The number of NC infused for cord blood transplants (CBT) is about one-tenth the NC in a standard allo-BMT. The CD 34^+ cells in UCB is about the same as in adult BM. We could assume that the minimum $CD34^+$ cell number for engraftment in CBT is 2×10^5/kg. This would be in agreement with the assumption that the primitive progenitors are present at about an 8-fold higher frequency in UCB than in adult BM (17). There were only 2 UCB samples in our 24 collections (<10%) that contained sufficient $CD34^+$ cells for engrafting a recipient weighing > 50 kg when 2×10^5 $CD34^+$ cells/kg is used as the threshold.

An average 103.32×10^6 $CD34^+$ cells could be obtained from an initial 1.72×10^6 UCB-derived $CD34^+$ cells after 10–14 days of expansion in our culture system. Some of the long-term marrow repopulating cells that currently cannot be tested may be recruited into the cell cycle and impair the long-term reconstitution potential. Thus, we increased the threshold $CD34^+$ cell number to 1×10^6/kg, that is, 4–5-fold more than the actual $CD34^+$ cells infused in unmanipulated CBT. Sufficient $CD34^+$ cells for transplanting adults would then be available in all but 1 of the 8 tested UCB samples (87.5%) after 10–14 days of expansion in our culture system. Therefore, it would be possible for most UCB units to be used for transplantation in adult patients after ex vivo expansion, thus expanding the UCB donor pool.

The repopulating potential is most critical for clinical use of ex vivo manipulated HPC (6,22–24). However, because of the lack of a suitable assay system for evaluation of the hematopoietic potential of candidate human HSC, the functional characteristics of the ex vivo expanded HPC are still incompletely understood (49). Early experimental attempts at expanding purified populations of HSC have generally led to a progressive loss of self-renewal capacity, coupled with increasing lineage commitment (50). Many investigations have indicated that the newly cloned early acting cytokines FL and TPO might be helpful in solving this problem (6,14,22–24). More recently, several culture systems containing the combination of any two of the three early acting cytokines, SCF, FL, and TPO, have been described that allow a substantial expansion of primitive HPC, such as LTCIC and the $CD34^+CD38^-$ subpopulation (10,11,14,26–28). A few studies using severe combined immunodeficiency (SCID) mice or fetal sheep as an in vivo model have shown the capacity of ex vivo expanded HPC to contribute to long-term in vivo hematopoiesis (51,52). Clinically, ex vivo expanded autologous PBSC given alone have show excellent PB recovery in patients pretreated with nonmyeloablative chemotherapy protocols (53). In contrast, when expanded autologous PBSC alone were infused into myeloablated patients, graft failure occurred (54). There has been no report on using expanded HPC alone for allogeneic transplantation. It still remains uncertain, therefore, if total hematopoietic reconstitution can be obtained by transfusion of expanded stem cells (21,23).

The in vitro characteristics of the expanded UCB hematopoietic cells were evaluated in three aspects in this study. First, the expansion of all progenitor cell compartments, including primitive LTCIC and $CD34^+CD38^-HLA\text{-}DR^-$ subpopulations, were evaluated to determine if expanded UCB cells still contain cells with long-term repopulating potential. Our results indicate that primitive LTC-IC and $CD34^+CD38^-HLA\text{-}DR^-$ subpopulations not only were maintained but also were substantially amplified during the culture. Second, the immunophenotypic profile ($CD34^+$ cells and subpopulations) was tracked throughout this study as an indicator of cellular composition of various populations. Of the $CD34^+$ cells, the primitive progenitor subpopulations of $CD38^-HLA\text{-}DR^-$, $CD38^-HLA\text{-}DR^+$, and $CD38^+HLA\text{-}DR^-$ were maintained or increased, but the proportions of the three subpopulation of $CD34^+$ lymphocytic precursors were significantly decreased compared with their initial levels, which may affect immunologic reconstitution after transplantation. Finally, to further evaluate the in vitro characteristics of the expanded UCB cells, reselection of $CD34^+$ cells from 4 expanded UCB products on day 14 of expansion were evaluated and compared for clonogenic efficiency, $CD34^+$ subpopulations, and expansion potential of the expanded $CD34^+$ cells with the initial $CD34^+$ UCB cells. The results indicated that the ex vivo-expanded $CD34^+$ cells retained in vitro characteristics of the initial fresh $CD34^+$ cells, including the clonogenicity, expansion potential, and composition of primitive $CD34^+$ subpopulations. These results indirectly demonstrated that the expanded UCB hematopoietic progenitors sustain the repopulating potential.

In conclusion, our data clearly demonstrate that our short-term culture system, consisting of defined QBSF-60 serum-free medium with a simple early acting cytokine combination of SCF, FL, and TPO, could substantially support the simultaneous expansion of all progenitor cell com-

partments, from very primitive $CD34^+CD38^-HLA\text{-}DR^-$ subpopulations and LTCIC, $CD34^+$ cells and HPP-CFC, to more mature committed progenitors (CFU-C) in UCB-derived $CD34^+$ cells. The expanded UCB HPC sustain the repopulating potential. If the process supports engraftment in the SCID mice or sheep model, the protocol would be clinically applicable for ex vivo expansion of human UCB-derived $CD34^+$ cells.

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Concise Review

Serum-Free Culture of Hematopoietic Stem Cells: A Review

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Key Words. Serum-free • Cell culture • Stem cells

Abstract. The development of serum-free systems for the maintenance and expansion of both primitive and committed hematopoietic progenitors has numerous applications in both basic and clinical research. Many different media have been tested and refined over the years, and current formulations now yield results similar to those observed with fetal bovine serum-based medias. Using these serum-free culture systems, the impact of the cell microenvironment and individual growth factors on primitive and maturing stem cells have both been studied. The utility of progenitor populations expanded ex vivo under serum-free conditions is under investigation.

Introduction

Initial attempts to culture and characterize the growth properties of hematopoietic progenitors were performed in a medium containing a variety of animal-based sera. However, the variability of the sources, collection and processing procedures for these sera produced considerable lot-to-lot compositional variability, necessitating elaborate screening procedures to assure consistent performance of cultures. Such use of an undefined medium also complicates the identification of the multiple interactions and pathways which control the proliferation, differentiation and senescence of these cells. In order to overcome these drawbacks, a number of investigators have developed serum-free media for the culture of both hematopoietic stem cells and progenitors. This review describes the historical development of serum-free media for the culture of hematopoietic cells and their use in experiments to characterize the interaction of stem cells with both soluble and stromal factors in their environment. Last, we discuss current serum-free culture systems for the expansion of hematopoietic progenitors and their use in therapeutic applications.

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Development of Serum-Free Medium for Hematopoietic Cells

The first studies to replace serum in hematopoietic cultures relied on components from serum-free cultures used to support non-hematopoietic cells [1]. In these studies, combinations of hormones, nutrients and attachment factors were used to stimulate hematopoietic cell growth and differentiation. In 1976, *Guilbert* and *Iscove* [2] reported that selenite, transferrin, albumin and lecithin could partially substitute for serum in the culture of granulocyte-macrophage progenitors (CFU-GM) and colony forming units-erythroid (CFU-E). Subsequently, a variety of modifications have been made to these recipes to enhance colony formation in the complete absence of serum. *Iizuka* and *Murphy* [3] demonstrated that transferrin was essential for the serum-free culture of CFU-GM, while *Cormier et al.* [4] reported that the addition of bovine serum albumin, iron saturated transferrin, soybean phospholipids, cholesterol, hemin, erythropoietin (EPO) and a partially purified extract of spleen conditioned medium called burst promoting activity to Iscove's modified Dulbecco's medium (IMDM) led to enhanced growth of murine burst forming units-erythroid (BFU-E) and multipotential progenitors. In addition, a similar medium containing bovine pancreatic insulin was developed by *Eliason* and *Odartchenko* [5]. Using this medium, 90% of colonies supported by the serum cultures were maintained without serum and the importance of EPO in the growth of erythroid colonies was reconfirmed.

Many investigators have published studies which define serum-free culture conditions for human hematopoietic progenitors [5-10]. *Sonoda* and *Ogawa* [11] reported an "attenuated quarter strength" α-medium which, when used at reduced 5% oxygen tension, supported the proliferation of single and multilineage hematopoietic colonies, even at low cell densities. The number of colonies produced was a linear function of the number of cells plated. Subsequently, *Drouet et al.* [12] described a serum-free liquid culture system to maintain progenitors for at least four weeks. In these cultures, IMDM was supplemented with bovine serum albumin, human transferrin, bovine insulin, soybean lecithin, cholesterol, hydrocortisone and alpha-thioglycerol, and used to culture human bone marrow for extended periods. Although both myelopoiesis and erythropoiesis steadily declined, there were no statistical differences in the number or type of colonies produced in the serum-free or serum-containing cultures.

Serum-Free Culture and Growth Factor Interactions

Using serum-free conditions similar to those described above, multiple studies have been performed to elucidate the effects of different growth factors on hematopoietic stem and progenitor cells. In early studies [13-15], conditioned media, tissue extracts and partially purified fractions were tested for their stimulatory activity on mouse or human bone marrow. With the availability of recombinant growth factors, several groups began studies to characterize the interactions of individual cytokines on progenitor activity. G-CSF and GM-CSF were found to stimulate cells of the myeloid lineage, although the proportion of cells driven toward the macrophage or neutrophil lineages differed in serum-free and serum-containing cultures [16-18]. Likewise, CSF-1 was confirmed to stimulate predominantly macrophage colonies [8]. Many subsequent studies focused on the identification of earlier acting growth factors which modulated the activity of more primitive progenitors. Interleukin 3 (IL-3) proved synergistic with other more lineage-restricted growth factors, promoting more colony growth than either growth factor alone [8, 19-22]. In these studies, IL-3 appeared to stimulate multipotent targets which were at earlier stages of development. However, invariably, IL-3 in combination with the late-acting factors induced the terminal differentiation of the cultures [20, 21].

More recently, serum-free cultures have been used to elucidate the actions of stem cell factor (SCF), the c-*kit* ligand, on pluripotent stem cells. In several reported studies, SCF synergized with IL-3 or other early-acting growth factors to enhance even further progenitor proliferation together with myeloid, megakaryocyte and erythroid hematopoiesis [23-27]. However, in some cases, greater numbers of growth factors were required together with SCF in serum-free cultures to produce colony activity equivalent to that observed in serum-containing cultures [23].

Several studies using serum-free culture have also probed the specific interactions of recombinant growth factors on highly purified progenitors or stem cells [28-30]. In studies using isolated bone marrow $CD34^+$ $HLA\text{-}DR^+$ c-kit^+ cells, IL-3 promoted the survival of progenitors, whereas SCF alone preferentially sustained more primitive pre-CFUs and delayed the DNA fragmentation associated with apoptosis [27]. In contrast, the cytokines, transforming growth factor β1 (TGF-β1), the chemokines, macrophage inflammatory protein-1α (MIP-1α), MIP-2β, platelet factor 4, IL-8 and macrophage chemotactic and activating factor (MCAF), along with H-ferritin had distinct suppressive effects on bone marrow $CD34^+$ cells [28]. In studies using cord blood stem cells, combinations of growth factors were found to selectively expand erythroid or myeloid progenitors from $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells [29, 30]. In these studies, SCF, IL-6, IL-3 and EPO induced maximal expansions of erythroid progenitors, whereas growth factor combinations lacking EPO but containing PIXY321 and G-CSF yielded maximal increases in myeloid precursors. The inhibitory growth factors found to suppress bone marrow $CD34^+$ cells had reduced effects on cord blood $CD34^+$ cells [28]. Finally, as the ultimate test of the maintenance of stem cells in serum-free culture, engraftment studies examining the repopulating activity of murine wheat germ agglutinin $(WGA^+)SCA^+Lin^-$ cells were performed [31]. The results indicated that 67% of long-term reconstitutive activity of these primitive cells could be maintained for two weeks in serum-free culture containing SCF, IL-6 and EPO, and that the preservation of the reconstituting cells did not correlate with the expansion of cells with the $WGA^+SCA^+Lin^-$ phenotype [31].

Properties of Stem Cells in Serum-Free Culture

The study of purified stem cells in serum-free culture has led to further understanding of the dynamics of these cells in their microenvironment. In long-term erythroid cultures, *Lansdorp* and *Dragowska* [32] found that serum-free cultures without cytokines maintained $CD34^+CD45RA^{lo}CD71^{lo}$ cell viability for longer periods than did serum-containing cultures. Furthermore, $CD34^+CD45RA^{lo}CD71^{lo}$ cell proliferation, together with the low turnover of dividing $CD34^+$ cells, contributed to the maintenance of the observed precursor cell number [33]. In further experiments where cord blood $CD34^+CD45RA^{lo}CD71^{lo}$ cells were subfractionated into $Thy1^+$ and $Thy1^-$ subsets, it was the $Thy1^+$ population that showed the highest (although transient) expansion potential [34]. Moreover, the proliferative potential of $CD34^+CD45RA^{lo}CD71^{lo}$ cells from adult bone marrow, umbilical cord blood and fetal liver were directly compared in serum-free culture [35]. In these experiments, the proliferative capacity of the primitive cells dramatically decreased with increasing age of the donor and suggested that the self-renewal and differentiation capabilities of primitive stem cells may change during ontogeny [35].

Several studies have examined the mechanisms of proliferation and differentiation of hematopoietic progenitors. Using IL-3-dependent hematopoietic cell lines transfected with the human *bcl*-2 gene, multilineage differentiation was studied [36]. In single cell serum-free culture, lineage commitment was observed without cell division, even in the absence of growth factors. Furthermore, *Mayani et al.* [37] used single cell culture of cord blood $CD34^+CD45RA^{lo}CD71^{lo}$ stem cells and studied the differentiation of the individual progeny. In 3-17% of the cell divisions, the individual progeny developed asymmetrically yielding cells with distinct functional activities. The remainder of the divisions were symmetric, producing similar progeny. These observations of both asymmetric and symmetric cell divisions suggested that early hematopoiesis and lineage commitment are regulated by a stochastic process independent of the known growth factors.

Future Clinical Application of Serum-Free Hematopoietic Progenitor Expansion

The culture of hematopoietic progenitor cells could have broad application in clinical medicine. Such expanded stem cell populations could provide hematologic support following high-dose or repetitive chemotherapy regimens, potentially reducing some of the side effects of these treatments. In addition, the culture of stem cells could serve to purge tumor cells from autografts. For allogeneic transplantation, cultured stem cells from donors could hasten engraftment, thereby minimizing the morbidity and mortality associated with prolonged cytopenias. Finally, stem cell expansion is crucial for many gene therapy protocols. In most cases, stem cell culture is essential for efficient gene transfer, mandating the development of methods to expand both primitive and committed stem cell populations.

The use of cultured stem cells as autologous transplants has been described in a limited number of patients [38]. In these instances, autologous bone marrow was maintained in conventional serum-containing cultures for short periods prior to infusion. To improve the reproducibility of these cultures, *Da et al.* [39] developed serum-free culture conditions to selectively maintain primitive hematopoietic activity while not supporting leukemic cell growth. In these studies, acute lymphoblastic progenitors (ALL-CFU) and polymerase chain reaction (PCR) for the *bcr/abl* fusion oncogene were used to track the survival of tumor cells in these cultures. In two of five experiments, PCR^+ leukemogenic cells disappeared after 3-4 weeks of culture. In all cultures, there was no evidence of leukemic cells despite an initial input of 75-100% blast forms, establishing the utility of serum-free culture for tumor purging.

In recent experiments in our laboratories, serum-free and serum-containing cultures were directly compared for the expansion of purified human bone marrow or peripheral blood $CD34^+$ cells [40, *Schain et al.*, unpublished data]. With IL-1, IL-3 and SCF, cell numbers increased approximately 80-fold over the first two weeks in culture, yielding 4- to10-fold higher numbers of cells than those in identical cultures containing fetal calf serum (Fig. 1). Upon flow cytometry analysis, the serum-free culture lacked detectable B and T cells, and became almost entirely $CD33^+$ after five days of culture. $CD34^+$ cell numbers increased 2- to 10-fold during the first 7-10 days of culture (Fig. 2). Likewise, $CD41a^+$ megakaryocyte precursors increased 50- to 700-fold during the first two

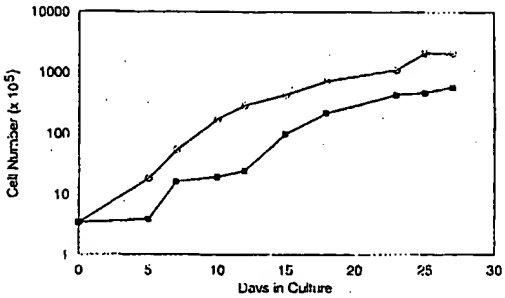

Fig. 1. Expansion of cell number. Isolated bone marrow CD34⁺ cells were cultured in serum-free or serum-containing culture using IL-1, IL-3 and SCF. Cell number was determined by hemocytometer counts performed periodically throughout the cultures. (○) = serum-free medium; (■) = serum-containing medium. CD34⁺ cells for each direct comparison experiment were isolated from individual pools of bone marrow. Each pool was composed of 25 ml of bone marrow from each of three to five separate donors. No donor contributed marrow to more than one pool. This experiment is representative of four direct comparison experiments performed.

weeks of serum-free culture. Parallel with the CD34⁺ cell expansion was a 5- to 35-fold expansion of CFU-GM (Fig. 3) and an 8- to 300-fold increase in BFU-E (Fig. 4). Expansions varied with the combination of growth factors used for stimulation. Maximal expansion of cells and progenitors was observed when G-CSF or GM-CSF

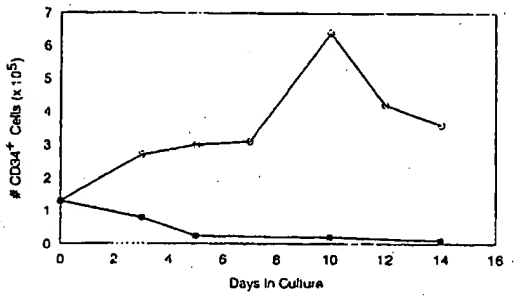

Fig. 2. Absolute number of CD34⁺ cells. The number of CD34⁺ cells was calculated as (% CD34⁺) × (# total cells). The number of CD34⁺ cells was determined for those cultures phenotyped in Figure 1 and is representative of results seen in four direct comparison experiments. (○) = serum-free medium; (■) = serum-containing mediums.

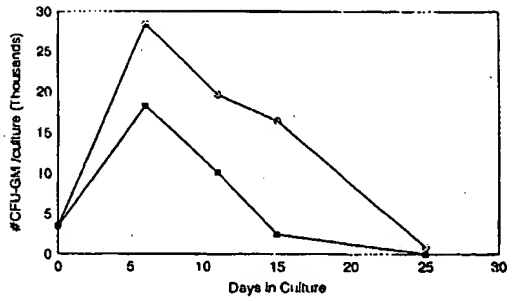

Fig. 3. CFU-GM content of cultures. Aliquots from the cultures described in Figure Legend 1 were assayed in methylcellulose culture for the content of CFU-GM at various time points. (○) = serum-free medium; (■) = serum-containing mediums.

was combined with SCF, IL-3 or IL-6 during culture. Clinical studies using such expanded populations as transfusion support to shorten cytopenic periods post-high- or repetitive-dose chemotherapy are planned.

Conclusions

The development of serum-free conditions for the culture of hematopoietic cells has facilitated the elucidation of some of the mechanisms which regulate the proliferation and differentiation of stem cells. Moreover, the reproducible serum-free expansion of hematopoietic progenitors and their differentiated progeny now permits clinical

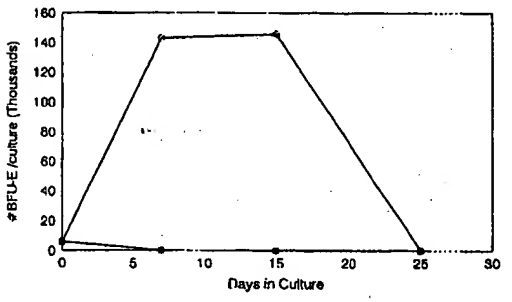

Fig. 4. BFU-E content of cultures. Aliquots from the cultures described in Figure Legend 1 were assayed in methylcellulose culture for the content of BFU-E at various time points. (○) = serum-free medium; (■) = serum-containing mediums.

studies of their use in hematologic support in a number of clinical settings. Further research defining the interactions of stem cells with the known and yet undiscovered growth factors and with the marrow microenvironment will be necessary to fully understand the processes of hematopoiesis and to begin to develop procedures to cultivate pluripotent stem cells.

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Mary Ann Liebert, Inc.

Ex Vivo Culture of Cord Blood CD34$^+$ Cells Expands Progenitor Cell Numbers, Preserves Engraftment Capacity in Nonobese Diabetic/Severe Combined Immunodeficient Mice, and Enhances Retroviral Transduction Efficiency

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ABSTRACT

Ex vivo **culture of hematopoietic stem/progenitor cells could potentially improve the efficacy of human placental/umbilical cord blood (CB) in clinical hematopoietic stem cell (HSC) transplantation and allow gene transduction using conventional retroviral vectors. Therefore, we first examined the effects of a 7-day period of *ex vivo* culture on the hematopoietic capacity of CB CD34$^+$ cells. Medium for the *ex vivo* cultures contain d either serum and six recombinant human hematopoietic growth factors (GFs), including Flt-3 ligand (FL), Kit ligand (KL = stem cell factor), thrombopoietin (Tpo), interleukin 3 (IL-3), granulocyte colony-stimulating factor (G-CSF), and interleukin 6 (IL-6), or a serum-free medium containing only FL, KL, and Tpo. After culture under both *ex vivo* conditions, the total numbers of viable cells, CD34$^+$ cells, colony-forming cells (CFCs), and long-term culture initiating cells (LTC-ICs) were increased. In contrast, the severe combined immunodeficiency (SCID) mouse engrafting potential (SEP) of cultured cells was slightly decreased, as compared with fresh cells. Nevertheless, cultured human CB CD34$^+$ cells were able to generate engraftment, shown to persist for up to 20 weeks after transplantation. We next tested the efficacy of retroviral transduction of cultured cells. Transduced cultured human cells were able to engraft in NOD/SCID mice, as tested 4 weeks after transplantation, and EGFP$^+$CD34$^+$ cells and EGFP$^+$ CFCs were isolated from the chimeras. Thus, although additional improvements in *ex vivo* culture are still needed to expand the numbers and function of human HSCs, the current conditions appear to allow gene transduction into hematopoietic SCID engrafting cells, while at least qualitatively preserving their *in vivo* engraftment potential.**

OVERVIEW SUMMARY

This study investigated improving *ex vivo* culture conditions for retroviral transduction of human hematopoietic stem cells (HSCs). Since successful hematopoietic gene therapy requires the transduced stem cells to engraft *in vivo*, we first evaluated the effects of *ex vivo* culture on the *in vivo* SCID engrafting potential (SEP) of cord blood (CB) CD34$^+$ cells. We found that the numbers of hematopoietic progenitor cells were increased after a 7-day period of *ex vivo* culture, and SEP was only slightly decreased. We then observed that human CB CD34$^+$ cells transduced during *ex vivo* culture in serum-free medium containing Flt3 ligand (FL), Kit ligand (KL = stem cell factor), and thrombopoietin (Tpo), generated EGFP$^+$CD34$^+$ cells and EGFP$^+$ CFCs in NOD/SCID chimeras. Thus, *ex vivo* culture allowed efficient gene transfer into human hematopoietic stem/progenitor cells, while preserving their *in vivo* engraftment potential.

INTRODUCTION

HUMAN UMBILICAL/PLACENTAL CORD BLOOD (CB) is now in active use as an alternative transplant graft source because of its advantages over bone marrow, including immediate availability of cryopreserved CB, lower risk of transmission of viral diseases, and reduced risk of graft-versus-host disease (GVHD) (Kurtzberg *et al.*, 1996; Cairo and Wagner, 1997). An

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additional advantage of CB is the potentially higher susceptibility of CB stem/progenitor cells to retroviral gene transfer (Lu *et al.*, 1993); CB may be a favorable source of "early" or more proliferative hematopoietic stem cells (HSCs). Successful gene transduction by retroviral vectors requires integration of the retroviral insert into cellular DNA, which in turn requires cell proliferation (Miller *et al.*, 1990). Thus, CB has been cultured *ex vivo* to increase HSC proliferation and thereby enhance retroviral transduction. Promising clinical results of retroviral transduction have already been achieved with target cells from CB (Lu *et al.*, 1993; Kohn *et al.*, 1995, 1998), and any significant enhancement of transduction efficiency could provide clear clinical benefit for use in selected inherited diseases including thalassemia, sickle cell anemia, Fanconi anemia, severe combined immunodeficiency (SCID) secondary to adenosine diaminase (ADA) deficiency, and a variety of metabolic/storage deficiencies (Kohn *et al.*, 1995; Cairo and Wagner, 1997; Lorincz *et al.*, 1997).

Ex vivo culture of hematopoietic cells has utilized hematopoietic growth factors (GFs) (Coutinho *et al.*, 1990; Brugger *et al.*, 1993; Srour *et al.*, 1993; Henschler *et al.*, 1994; McKenna *et al.*, 1995; Petzer *et al.*, 1996b; Conneally *et al.*, 1997; Prosper *et al.*, 1997; Zandstra *et al.*, 1997), with or without bone marrow stromal cells (Koller *et al.*, 1996; Breems *et al.*, 1998). Several groups have reported that *ex vivo* culture results in increased numbers of viable cells, $CD34^+$ cells, and colony-forming cells (CFCs), including long-term culture initiating cells (LTC-ICs) (Moore and Hoskins, 1994; Koller *et al.*, 1996; Petzer *et al.*, 1996b; Bhatia *et al.*, 1997; Conneally *et al.*, 1997; Piacibello *et al.*, 1997; Zandstra *et al.*, 1997; Luens *et al.*, 1998). Several combinations of GFs have been investigated, and combinations that include Flt-3 ligand (FL), Kit ligand (KL), and thrombopoietin (Tpo) have provided promising results (Conneally *et al.*, 1997; Luens *et al.*, 1998). Results of two clinical trials indicate that *ex vivo* cultured $CD34^+$ cells can be safely transplanted after high-dose chemotherapy, although the kinetics of bone marrow (BM) reconstitution have not yet been shown to be enhanced (Brugger *et al.*, 1995; Alcorn *et al.*, 1996; Emerson, 1996). More recently, the availability of immunodeficient animal hosts for human hematopoiesis has provided an additional method to test human hematopoietic cell populations for the ability to generate extensive multilineage hematopoiesis *in vivo* (Civin *et al.*, 1996; Larochelle *et al.*, 1996; Bhatia *et al.*, 1997; Conneally *et al.*, 1997; Hogan *et al.*, 1997; Cheng *et al.*, 1998; Leung *et al.*, 1998; Luens *et al.*, 1998; Ramírez *et al.*, 1998; Güenechea *et al.*, 1999; Piacibello *et al.*, 1999).

Therefore, we first performed quantitative comparisons of *ex vivo* cultured versus fresh CB $CD34^+$ cell populations in hematopoietic assays, including their ability to engraft at high levels in the nonobese diabetic/LtSz-*SCID/SCID* (NOD/SCID) hematopoietic chimera model. After finding that human CB $CD34^+$ cell populations cultured *ex vivo* for 7 days retained the ability to engraft in the NOD/SCID chimera model, we evaluated the efficiency of gene transduction of CB $CD34^+$ cells during culture in serum-free medium containing FL, KL, and Tpo. Our results indicate that *ex vivo* serum-free culture of CB $CD34^+$ cells allows efficient gene transduction of *in vitro* colony-forming cells (CFCs) and *in vivo* NOD/SCID engrafting cells.

MATERIALS AND METHODS

Experimental design

Freshly thawed (previously purified and cryopreserved) human CB $CD34^+$ cells were suspended in a given quantity of tissue culture medium containing GFs. Approximately half the volume of this "fresh" control cell suspension was assayed immediately for content of viable cells, $CD34^+$ cells, CFCs, LTC-ICs, and SCID mouse engrafting potential (SEP). The other "experimental" half of these cells was cultured *ex vivo* for 7 days before assessment by the same hematopoietic assays. Although cell counts were determined both before and after the 7-day *ex vivo* culture period, the input of *ex vivo* cultured cells into the hematopoietic assays was based on volume, rather than cell number. In this way, we directly compared the function of a given number of uncultured "fresh" control cells versus the experimental population of day 7 *ex vivo* "cultured" cells generated by that number of input day 0 fresh cells. Finally, we evaluated the efficiency of retroviral gene transduction (as assessed by marker gene expression in *in vitro* and *in vivo* assayed hematopoietic progeny) of cultured cells.

Human cells

We purchased cryopreserved CB $CD34^+$ cells from Poietic Technologies (Gaithersburg, MD) or PureCell, LLC (San Mateo, CA). $CD34^+$ cells were purified by immunomagnetic selection (Miltenyi Biotechnologies, Auburn, CA), then cryopreserved in Iscove's modified Dulbecco's medium (IMDM; HyClone Laboratories, Logan, UT) containing 50% fetal bovine serum (FBS; StemCell Technologies, Vancouver, BC, Canada) and 15% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO). After thawing, $89 \pm 5\%$ of the cells were viable (trypan blue dye exclusion), and $91 \pm 4\%$ of the cells were $CD34^+$ (Trischmann *et al.*, 1993).

Human stromal cells were prepared for LTC-IC assays from BM samples collected from the posterior iliac crests of consenting healthy adult volunteers under an Institutional Review Board (IRB)-approved protocol.

Recombinant human cytokines

Purified recombinant human KL, granulocyte colony-stimulating factor (G-CSF), and interleukin 2 (IL-3) were generously donated by Amgen (Thousand Oaks, CA). IL-6, FL, and Tpo were purchased from either R&D Systems (Minneapolis, MN) or PeproTech (Rocky Hill, NJ).

Antibodies and FACS analysis

Phycoerythrin (PE)-conjugated purified mouse anti-human CD34, CD19, and CD33, and PE-conjugated and fluorescein isothiocyanate (FITC)-conjugated isotype control antibodies were purchased from Becton Dickinson (San Jose, CA). FITC-conjugated rat anti-mouse CD45 and mouse-anti human CD3, and PE-conjugated mouse anti-human CD45 were purchased from Sigma. FITC-conjugated mouse anti-human CD13 was purchased from Dako (Carpinteria, CA). FITC-conjugated mouse anti-human CD41a, PE-Cychrome 5 (PE-CY5)-conjugated mouse anti-human CD45, and PE-CY5-conjugated iso-

type control were purchased from PharMingen (San Diego, CA).

A FACSort (Becton Dickinson) flow cytometer equipped with an Argon laser tuned at 488 nm was used for fluorescence-activated cell sorting (FACS) analysis. Green fluorescence from enhanced green fluorescent protein (EGFP) was detected in the FL1 emission channel (Cheng *et al.*, 1997, 1998).

Ex vivo *hematopoietic cultures*

CB CD34+ cells were plated at 5–20 × 10^4 cells/ml in 75-cm^2 flasks (Becton Dickinson) in either condition 1 (IMDM supplemented with 10% FBS and the following six recombinant humans GFs:FL [100 ng/ml], KL [100 ng/ml] Tpo [20 ng/ml], IL-3 [20 ng/ml], IL-6 [20 ng/ml], and G-CSF [20 ng/ml]) or condition 2 (QBSF-60 serum-free medium [Quality Biological, Gaithersburg, MD] containing the following three recombinant human GFs: FL [100 ng/ml], KL [100 ng/ml], and Tpo [20 ng/ml]). These suspension cultures were incubated in a 5% CO_2 humidified atmosphere at 37°C. In experiments 1–6 (Tables 1 and 2), at ~3.5 days, an equal volume of the same culture medium (either condition 1 or 2) was added to cultures. These cell cultures were then incubated for an additional ~3.5 days (for a total of 7 days of *ex vivo* culture). In the gene transduction experiments, the cells were resuspended onto RetroNectin with retroviral supernatant, as indicated for each single experiment in the footnotes to Tables 3–6.

Viable cell counts and CD34+ cell content

Fresh and cultured cells were counted with a Coulter Z1 cell counter (Coulter Diagnostics, Miami, FL). Viability was assessed by trypan blue dye exclusion and microscopy, then confirmed by LDS-751 (Exciton, Dayton, OH) dye exclusion and flow cytometry (Terstappen *et al.*, 1988). Immunophenotype was determined by multicolor flow cytometry after immunostaining (Trischmann *et al.*, 1993).

Progenitor cell assays

Colony-forming cell-mix (CFC-Mix), colony-forming cell-granulocyte/monocyte (CFC-GM), and burst-forming unit-erythroid (BFU-E) were evaluated by plating 500–1000 fresh CD34+ cells and the equivalent volume of cultured cells (see Experimental Design, above) in 1 ml of MethoCult GF H4434 methylcellulose medium (StemCell Technologies) in triplicate. Total and green fluorescent colonies were counted after 2 weeks, as described (Cheng *et al.*, 1998; Götze *et al.*, 1998). Individual colonies were subsequently picked from some plates and lysed for polymerase chain reaction (PCR) analysis (Cheng *et al.*, 1997).

CFC assays were also performed on cells recovered from the BM of NOD/SCID mice, using previously described methods shown to be selective for the growth of human colonies in a murine cell background (Carow *et al.*, 1993; Leung *et al.*, 1998; Ramírez *et al.*, 1998).

We quantitated the number of LTC-ICs in the fresh and cultured CB CD34+ cell populations by limiting dilution analysis as described (Sutherland *et al.*, 1990). For each evaluation, four concentrations of fresh cells and equivalent volumes of the cultured cell (see Experimental Design, above) were seeded in 20 replicates in 96-well plates containing preestablished adherent stromal cell layers and 200 μl of MyeloCult H5100 (StemCell Technologies) supplemented with 10^{-6} *M* freshly dissolved hydrocortisone. After 5 weeks of culture with medium refeeding as described above, adherent and nonadherent cells were harvested and pooled, then plated in 24-well plates for CFC assays.

Transduction of fresh and cultured cells

Amphotropic retroviral supernatants of the EGFP-encoding MGIN retroviral vector (derived from the murine stem cell virus [MSCV]) were produced in a human 293T packaging cell line (Cheng *et al.*, 1997). We performed gene transduction either by centrifugation ("spinoculation") or by incubation on fibronectin fragment CH-296 (RetroNectin; PanVera, Madison, WI)-coated surfaces.

Analysis of retroviral transduction by PCR

The efficiency of gene transfer into human progenitor cells was measured by PCR analysis of CFC colonies derived from CD34+ cells. Nested PCR for the EGFP gene was carried out on individual colonies picked from methylcellulose cultures. PCR amplification of a human endogenous DNA sequence was used as a control, as described (Ramírez *et al.*, 1998).

Transplantation of human cells for measurement of SEP

Mice were bred and maintained under pathogen-free conditions, as approved by the Animal Care Committee of the Johns Hopkins Medical Institutions (Ramírez *et al.*, 1998). Fresh or equivalent volumes of *ex vivo* cultured human cells (see Experimental Design, above) were transplanted by tail vein injection into sublethally irradiated (300 cGy, using a ^{137}Cs γ-irradiator) 6- to 8-week-old NOS/SCID mice. Mice were sacrificed 2–20 weeks after transplantation of human cells, as described for each experiment. Single-cell suspensions were prepared from the spleens, and BM cells were flushed from the removed femurs and tibias (Ramírez *et al.*, 1998). Cells were counted and viability determined (89 ± 3%). Because these four bones have been shown to contain ~25% of the total BM of a mouse (Boggs, 1984), the total number of BM cells per mouse was estimated by multiplying the number of cells obtained by 4.

Human cells in the BMs and spleens of the human–mouse chimeras were enumerated and cell lineages determined by three-color flow cytometry of cells immunostained with murine monoclonal antibodies, as described previously (Leung *et al.*, 1998; Ramírez *et al.*, 1998). SEP was defined as the (average) number of human CD45+ cells recovered from the BM of an experimental group of NOD/SCID mice per human CD34+ cell initially transplanted (Leung *et al.*, 1998).

Statistical analysis

We used a multiple linear regression model to compare the engraftment potential of the cultured versus fresh cells in experiments 1–5 (Leung *et al.*, 1998). Briefly, the dose–response relationship between the number of human hematopoietic cells transplanted and the number of human CD45+ cells in the BM of the chimera was determined by plotting the two variables

and then calculating the Pearson correlation coefficient. SEP was defined as the estimated slope of the resulting linear regression line, which represented the average number of human $CD45^+$ cells detected divided by the number of human $CD34^+$ cells transplanted.

We used single-hit Poisson statistics to analyze LTC-IC frequency and number in fresh or cultured cell populations (Sutherland *et al.*, 1990).

RESULTS

The numbers of total cells and $CD34^+$ cells are increased after ex vivo *culture*

Fresh cells were 89 ± 5% viable and cultured cells were 82 ± 8% viable. As shown in Table 1, the number of viable cells increased by more than 40-fold after ex vivo culture in either condition 1 (serum-containing medium plus six GFs: experiments 1–4) or condition 2 (serum-free medium plus three GFs: experiments 5 and 6). The frequency of $CD34^+$ cells fell from a mean of 91% in the fresh cells to 18% in the cultured cells (Fig. 1). Nevertheless, the total numbers of $CD34^+$ cells increased by an average of 9- to 10-fold after *ex vivo* culture (Table 1). In two experiments using condition 1, the cultured cells were also immunostained for the expression of several leukocyte differentiation antigens (Trischmann *et al.*, 1993). $CD13^+$ (myelomonocytic) cells comprised 70–80% of the cells, and $CD41a^+$ (megakaryocytic) cells comprised 5–10% of the cells. No $CD19^+$ (B lymphoid) or $CD3^+$ (T lymphoid) cells were detected (data not shown).

The numbers of CFCs are increased after ex vivo *culture*

On average in experiments 1–5, 1000 fresh CB $CD34^+$ cells generated 22 CFC-Mix, 52 CFC-GM, and 71 BFU-E colonies. In comparison, 1000 *ex vivo* cultured cells generated one CFC-Mix, seven CFC-GM, and seven BFU-E colonies. Because of the increase in total cell numbers during the *ex vivo* culture, the total numbers of CFCs in the entire cell population after 7 days in culture condition 1 increased by twofold for CFC-Mix, sixfold for CFC-GM, and fourfold for BFU-E (Table 1). These results were similar (and not statistically different) for culture condition 2.

Numbers of LTC-ICs after ex vivo *culture*

LTC-IC assays were performed in only three experiments (experiments 2, 3, and 5). The frequency of LTC-ICs was on

TABLE 1. EFFECTS OF 7-DAY *ex Vivo* CULTURE OF CORD BLOOD $CD34^+$ CELLS ON HEMATOPOIETIC CAPACITY: EXPERIMENTS 1–6[a]

Culture condition[b]	*Fold increase in viable cells*	*Fold increase in $CD34^+$ cells*	*Fold increase in CFCs*			*Fold increase in LTC-IC*[c]	*Fold increase in SEP*
			CFC-Mix	*CFC-GM*	*BFU-E*		
1. IMDM + FBS + FKT36G (experiments 1–4)	43.2 ± 12.5	9.7 ± 8.0	1.5 ± 0.3	6.2 ± 0.6	4.1 ± 2.5	2.2 ± 0.3	0.3 ± 0.2
2. QBSF-60 + FKT (experiments 5 and 6)	44.4 ± 18.5	8.8 ± 2.6	5.6 ± 3.9	6.1 ± 1.7	5.1 ± 2.0	1.3	0.9 ± 0.6

[a]Values represent the mean ± standard error increase in the total numbers of viable cells, $CD34^+$cells, CFCs, LTC-ICs, and SEP of 7 day-cultured CB $CD34^+$cells relative to fresh cells. Because large numbers of cells were needed for experiments 1–5, cells were pooled from three or four donor CB samples for each of these experiments; this also minimizes effects due to variability among donors in stem/progenitor cell number and function (Leung *et al.*, 1998). In experiments 6–10, cells from only one or two CB donors were pooled, due to the smaller size of the experiments. In experiments 1–6, mice received intraperitoneal injections of the following growth factors three times per week posttransplantation: IL-3, GM-CSF, G-CSF, and KL (10 μg of each/dose) combined in a single injection for each mouse, in order to maximize levels of human cells in the engrafted human–mouse chimeras (Leung *et al.*, 1998). In experiments 1–5, three doses of fresh and cultured cells were transplanted. This design enabled us to obtain easily detectable levels of human cell engraftment and dose–response curves for analysis using a multiple linear regression model (Leung *et al.*, 1998). In experiment 6, a single dose of fresh versus the equivalent volume of cultured cells was transplanted. In experiment 6, the probability of a significant difference in human $CD45^+$cell levels in the human–mouse chimeras was determined by Wilcoxon scores (rank sums) test.

[b]Experiments 1–4 were conducted in culture condition 1 (IMDM + 10% FCS + FL, KL, Tpo, IL-3, IL-6, and G-CSF). Experiments 5 and 6 were conducted in culture condition 2 (QBSF-60 + FL, KL, and Tpo). $CD34^+$cells were plated at the following concentrations: experiment 1, 1×10^5; experiment 2, 1.4×10^5; experiment 3, 1.6×10^5; experiment 4, 9×10^4; experiments 5–8, 5×10^4.

[c]LTC-IC assays were performed in only two experiments using culture condition 1, and in one experiment using culture condition 2, as described in Materials and Methods. For preparation of allogeneic stromal cells for the LTC-IC assays, MNCs were isolated by density-gradient centrifugation (Ficoll-Hypaque; Pharmacia Biotech, Piscataway, NJ) and suspended in myeloid long-term culture medium (H5100; StemCell Technologies) supplemented with 10^{-6} *M* water-soluble hydrocortisone (Sigma). Cells were plated onto 75-cm^2 tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ) and incubated at 37°C in a humidified 5% CO_2 atmosphere with weekly culture refeeding (Gartner and Kaplan, 1980). When confluent stromal cell layers were present (3–4 weeks after initiation of the cultures), stromal cells were harvested and cryopreserved in DMSO-containing medium. Ten days before the initiation of an LTC-IC assay, stromal cells were thawed and subcultured in 96-well tissue culture plates (Becton Dickinson) at 100–150 cells/μl in the same stromal cell culture medium. Subcultured stromal cell layers were irradiated (1500 cGy, using a ^{137}Cs γ irradiator) 2–6 days prior to initiation of LTC-IC assays.

average 1 per 1000 fresh CB CD34$^+$ cells. After culture in condition 1, the frequency of LTC-ICs decreased on average to 0.05 per 1000 cultured cells. After culture in condition 2, the LTC-IC frequency decreased to 0.02 per 1000 cultured cells. The average total numbers of LTC-ICs in the cell populations after 7 days of *ex vivo* culture increased by twofold in condition 1 and remained essentially constant in condition 2 (Table 1).

Effect of ex vivo *culture on SEP*

A total of 173 sublethally irradiated NOD/SCID mice was transplanted with fresh or cultured human cells in experiments 1–6. As we and others have previously reported (Leung *et al.*, 1998; van der Loo *et al.*, 1998), a dose–response relationship between the numbers of CD34$^+$ cells transplanted and the numbers of human CD45$^+$ cells recovered from the BMs and spleens of the hematopoietic chimeras was observed overall (Figs. 2 and 3). Human cells were easily detected in all mice transplanted with fresh or cultured cells by flow cytometry. Up to 98 $\times$ 10^6 human CD45$^+$ cells were identified in the BMs of chimeras transplanted with fresh cells, and up to 39 $\times$ 10^6 human CD45$^+$ cells were identified in the BMs of the chimeras transplanted with cultured cells (Fig. 2). In experiments 1–4 (condition 1), and in experiment 5 (condition 2), the SEP was lower for cultured cells than for the corresponding fresh cells (Fig. 2, Tables 1 and 2). In experiments 3–5, we randomized mice to be sacrificed 2–3 or 7–20 weeks after transplantation; we operationally defined these lengths of follow-up as "short term" and "long term," respectively. In all cases, the cultured cells had lower SEP than the corresponding fresh cells (the range of the ratio of the SEP of cultured versus fresh cells was 0.1–0.7), even though the difference was not always significant (Table 2, Fig. 2). Experiment 6 was a smaller experiment performed with a single dose of fresh versus cultured cells, which prevented linear regression analysis. In this experiment, the average number of CD45$^+$ cells in the BMs of the NOD/SCID mice transplanted with fresh cells was 10.6 ($\pm$9) $\times$ 10^6, while the average number in the BMs of the animals transplanted with cultured cells was 18.2 ($\pm$12.5) $\times$ 10^6. This 1.7-fold increase in engraftment of the cultured cells versus fresh cells was not significant by Wilcoxon scores test ($p = 0.4$). Finally, for all these results taken together (both culture conditions, analyzed at both time points), 1 fresh CD34$^+$ cell was able to generate 38 CD45$^+$ cells in the BMs of the chimeras, while 1 cultured CD34$^+$ cell was able to generate 14 CD45$^+$ cells. Overall, the weighted average SEP ratio of cultured to fresh cells was 0.4; i.e., fresh cells had a 2.8-fold higher SEP ($p = 0.0002$) (Table 2). Figure 3 shows the combined regression fits for all 173 mice analyzed in our study.

All animals transplanted with fresh or cultured cells had human CD13$^+$, CD19$^+$, and CD34$^+$ cells in their BMs by flow cytometry (data not shown). As has been found previously in these human–mouse chimeras (Leung *et al.*, 1998; Ramírez *et al.*, 1998), no CD3$^+$ (T lymphoid) cells were detected. In experiments 2, 4, 5, and 6, the chimera BM cells were plated for CFC assays. Human myeloid and erythroid colonies were generated from all the chimeras transplanted with either fresh or cultured cells, assessed at short-time and long-term time points (data not shown). There was an overall dose–response relationship between the number of colonies recovered and the

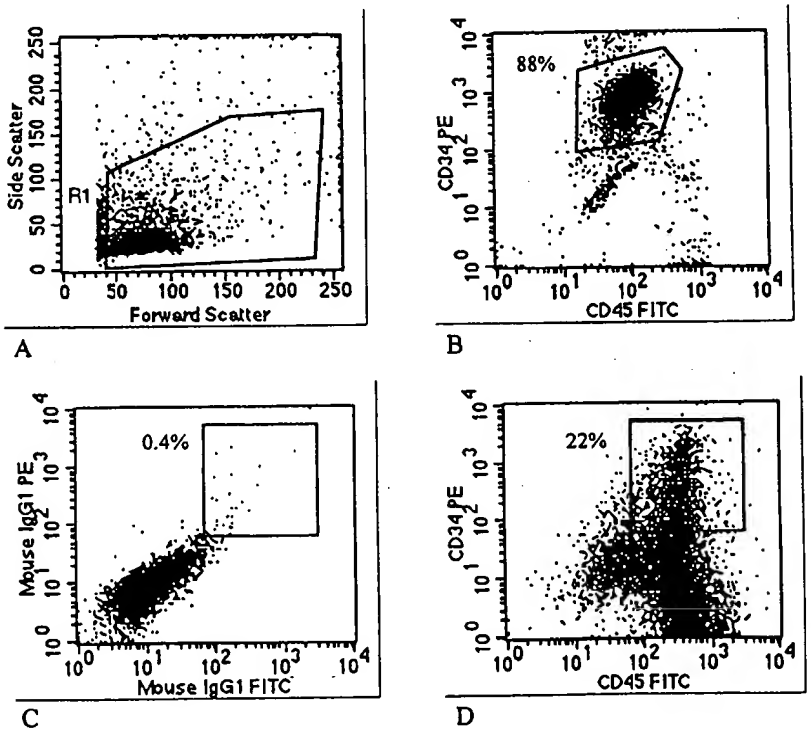

FIG. 1. CD34$^+$ cell frequency in fresh versus *ex vivo* cultured cells. Aliquots of fresh versus cultured cells were immunostained and analyzed by flow cytometry: (**A** and **B**) Fresh CD34$^+$ cells; (**C** and **D**) 7 day-cultured cells. Percentages of CD34$^+$ cells are shown. These results are from experiment 4 (Table 1) and are representative of all the experiments.

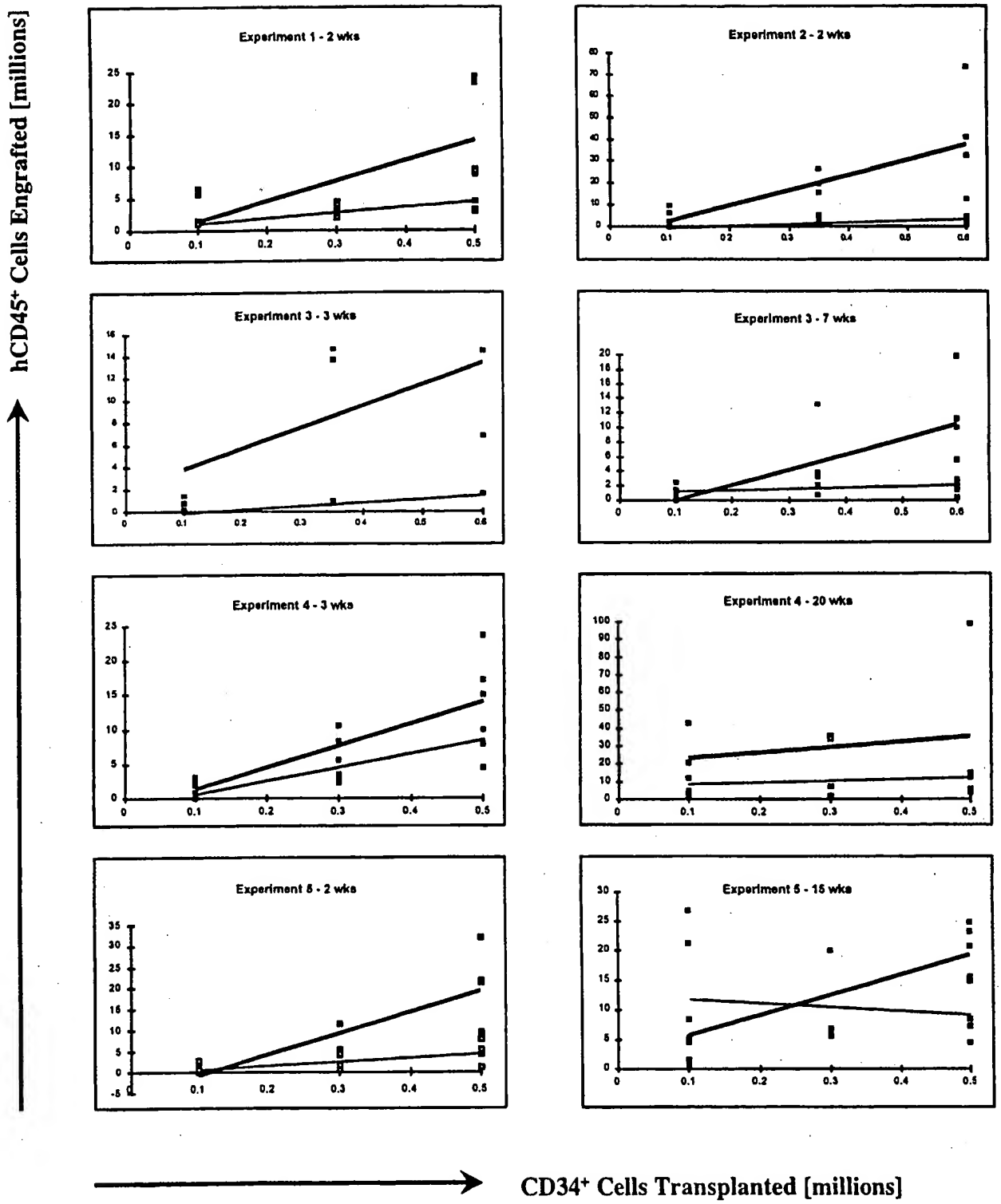

FIG. 2. SEP of fresh versus cultured cells. Three cell doses of fresh or cultured cells were transplanted into sublethally irradiated NOD/SCID mice in experiments 1–5 (Table 1). Individual experiments are indicated, along with the time point (weeks after transplant) at which the NOD/SCID chimeras were assayed for human cell content. The dose of $CD34^+$ cells transplanted is plotted on the x axis, and the number of human $CD45^+$ ($hCD45^+$) cells engrafted is plotted on the y axis. Data from each chimera transplanted with fresh cells (closed squares) or cultured cells (open squares) are shown. Each line represents the multiple linear regression fit for the data from the transplanted chimeras (fresh cells, thick line; cultured cells, thin line).

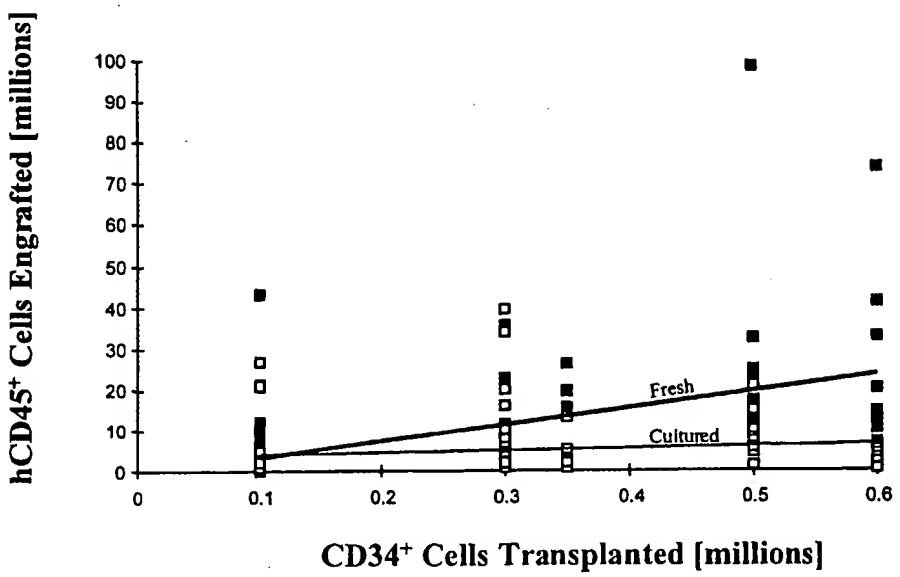

FIG. 3. SEP of fresh versus cultured cells. This multiple linear regression analysis includes data from all six experiments (n = 173; Table 1). The SEP of fresh cells was 37.9, and the SEP of cultured cells was 13.7. The difference between the two values was significant (p = 0.0002). Symbols and lines are as in Fig. 2.

TABLE 2. *Ex Vivo*-CULTURED CORD BLOOD CD34+ CELL POPULATION ENGRAFTMENT *in Vivo*, AND EFFECT ON SCID ENGRAFTMENT POTENTIAL: EXPERIMENTS 1–6

Culture condition[a]	*Experiment*	*Assay time point (weeks after transplant)*[b]	*SEP*			p *Value*	*No. of mice*
			Fresh	*Cultured*	*Cultured/fresh*[c]		
1. IMDM + FCS + FKT36G	1	2	27.5	10.3	0.4	0.06	23
	2	2	61.4	5.2	0.1	0.004	24
	3	3	23.2	2.6	0.1	0.04	12
		7	16.5	5.4	0.3	0.04	23
	4	3	26.9	16.7	0.6	0.4	23
		20	72.9	33.1	0.4	0.3	15
2. QBSF-60 + FKT	5	2	35.5	9.1	0.3	0.01	25
		15	39.4	26.9	0.7	0.006	21
	6	10	10.6	18.2	1.7	0.4	7
	1–6	2–20	37.9	13.7	0.4	0.0002	173

[a]Same abbreviations as in Table 1. The last row combines all data obtained under both culture conditions, and at both short- and long-term time points.

[b]NOD/SCID mice were sacrificed after 2–20 weeks, as indicated in text and in Fig. 2.

[c]Ratio of SEPs. We also analyzed the results of the experiments grouped by culture condition, time point of assay ("short term" or "long term"), and a combination of the two. In the experiments utilizing culture condition 1, the SEP of fresh cells was 38.2, the SEP of cultured cells was 10.1, and the weighted average SEP ratio (cultured:fresh) was 0.3 (p = 0.005, n = 0120 mice). In the experiments utilizing culture condition 2, the SEP of fresh cells was 37.0, the SEP of cultured cells was 22.9, and the weighted average SEP ratio (cultured:fresh) was 0.6 (p = 0.0002, n = 53 mice). In the experiments utilizing culture condition 1 analyzed at 2–3 weeks after transplant, the SEP of fresh cells was 38.7, the SEP of cultured cells was 8.7, and the weighted average SEP ratio (cultured:fresh) was 0.2 (p = 0.0001, n = 82 mice). In the experiments utilizing culture condition 1 analyzed at 7–20 weeks, the SEP of fresh cells was 37.1, the SEP of cultured cells was 13.2, and the weighted average SEP ratio (cultured:fresh) was 0.3 (p = 0.04, p = 38 mice). In the experiments utilizing culture condition 2 analyzed at 7–20 weeks, the SEP of fresh cells was 38.6, the SEP of cultured cells was 33.5, and the weighted average SEP ratio (cultured:fresh) was 0.9 (p = 0.01, p = 28 mice). For all the experiments in which the SEP was determined 2–3 weeks after transplant (including both culture conditions), the SEP of fresh cells was 38.1, the SEP of cultured cells was 8.8, and the weighted average SEP ratio (cultured:fresh) was 0.2 (p = 0.0001, p = 107 mice). For all the experiments in which the SEP was determined at 7–20 weeks after transplant (including both culture conditions), the SEP of fresh cells was 37.6, the SEP of cultured cells was 21.7, and the weighted average SEP ratio (cultured:fresh) was 0.6 (p = 0.3, p = 28 mice).

$CD34^+$ cell dose transplanted (data not shown). The data obtained from the spleens of the animals paralleled those obtained from the BM, although the levels of engraftment were lower (data not shown).

Ex vivo *culture enhances retroviral gene transduction of* in vitro *progenitor cells*

In experiment 7, we transduced either fresh CB $CD34^+$ cells or cells "precultured" (cultured *ex vivo* prior to transduction) in condition 2 for 2 days (the duration of preculture used in several published retroviral transduction protocols) (Larochelle *et al.*, 1996; Cheng *et al.*, 1997, 1998). In the cell population that had been transduced after 2 days of preculture, 12% of the $CD34^+$ cells and 19% of the $CD34^-$ cells were $EGFP^+$, by flow cytometry on day 7 (Table 3). In contrast, in the cell population that had been transduced without preculture, only 0.5% of the $CD34^+$ cells and 1.8% of the $CD34^-$ cells were $EGFP^+$. About 28% of CFCs transduced on day 2 expressed EGFP by fluorescence microscopy, while only ~8% of the CFC from cells transduced on day 0 were $EGFP^+$ (Table 3). PCR analyses of plucked colonies confirmed these results (Table 3).

In experiment 8, we compared gene transduction efficiency after three different periods of preculture (condition 2) and using three transduction strategies. Three to 24% of $CD34^+$ cells, $CD45^+$ cells, or $CD13/33^+$ cells expressed EGFP by flow cytometry. Each of these strategies resulted in efficient transduction, with up to 60% of $EGFP^+$ CFCs (Table 4).

In experiment 9, we explored the effects of multiple additions of retroviral supernatant to *ex vivo* cultures of CB $CD34^+$ cells incubated on RetroNectin. After *ex vivo* culture under these conditions for 7 days, with daily addition of retroviral supernatant from days 2 to 6, 52% of $CD34^+$ cells and 52% of CFCs were $EGFP^+$ (Table 5).

In experiment 10, we compared 4 days (total duration) of culture with addition of retroviral supernatant on days 2 and 3,

TABLE 3. ENHANCEMENT OF RETROVIRAL TRANSDUCTION EFFICIENCY IN 2-DAY CULTURE OF $CD34^+$ CELLS IN SERUM-FREE MEDIUM CONTAINING THREE GROWTH FACTORS, AS ASSESSED BY *in Vitro* ASSAYS: EXPERIMENT 7

Days in culture[a]	*Transduction*	*EGFP*$^+$					
		CD34^{+}[b]	*CD34*$^{-}$[b]	*CFC-Mix*[c]	*CFC-GM*[c]	*BFU-E*[c]	*CFC*[d]
0 + 7	Mock spinoculation	0	0	0 / 2 ± 1	0 / 28 ± 4	0 / 41 ± 4	0 / 10
	Spinoculation	0.5 ± 0.1	1.8 ± 0.2	0 / 2 ± 1	3 ± 2 / 34 ± 2	3 ± 1 / 37 ± 13	0 / 10
2 + 5	Mock spinoculation	0	0	0 / 2 ± 1	0 / 51 ± 12	0 / 55 ± 2	0 / 10
	Spinoculation	12 ± 0.3	19 ± 0.2	2 ± 2 / 5 ± 2	22 ± 6 / 54 ± 4	13 ± 6 / 74 ± 22	3 / 10

[a]In order to study the effects of *ex vivo* culture on gene transduction efficiency, we transduced fresh versus cultured (condition 2) CB $CD34^+$ cells with the EGFP-encoding MGIN retroviral vector, which was derived from the murine stem cell virus (MSCV) (Cheng *et al.*, 1997). The cells were transduced with the MGIN retrovirus by spinoculation for 4 hr. Briefly, in both experiments 7 and 8, freshly thawed retroviral supernatants from the packaging cell line were mixed 1:1 (v/v) in a 15-ml polypropylene tube (Becton Dickinson) with an aliquot of target cells in QBSF-60 containing FL, KL, and Tpo (GFs at the same concentrations as used for *ex vivo* cultures) and 8 mg/ml polybrene (Sigma). This transduction suspension was centrifuged at 1800 × *g* at 32–35°C for 4 hr. After this 4-hr "spinoculation," cells were washed once and immediately aliquoted for *in vitro* assays. In addition, in experiments 8–10, we followed the manufacturer recommendations for retrovirus-mediated gene transfer on RetroNectin. In brief, aliquots of freshly thawed retroviral supernatants from the packaging cell line were preincubated on RetroNectin-coated 6-well plates (Becton Dickinson) for 1 hr at room temperature. The cultured cell suspension and the remaining viral supernatant were then added to the plate. The transduction mixture was incubated for 2–3 days in a 5% CO_2 humidified atmosphere at 37°C. An aliquot was then plated for CFC assay, and the remaining cells were cultured under condition 2 for an additional 7 days (cells transduced on day 0), or 5 days (cells transduced on day 2) prior to assessment by flow cytometry. *0 + 7* indicates that $CD34^+$ cells were transduced on day 0 and cultured for an additional 7 days. *2 + 5* indicates that cells were transduced on day 2 and cultured for an additional 5 days.

[b]Percentage of $CD34^+$ and $CD34^-$ cells that were $EGFP^+$ by flow cytometry.

[c]Number of $EGFP^+$ (numerator) and total (denominator) CFCs were scored by fluorescence microscopy. Results are expressed as means ± standard error (five replicates).

[d]Ten CFCs were randomly plucked from the plates and tested for the presence of the EGFP transgene by PCR. Type of CFC was not noted. All EGFP PCR primers were within the EGFP cDNA: EGFP1 sense primer: 5′-GGAGAGGGTGAAGGTGATGC-3′; EGFP1 antisense primer: 5′-CCATGTGTAATCCCAGCAGC-3′; EGFP2 sense primer: 5′-CAAGAGTGCCATGCCC-GAAGG-3′; EGFP2 antisense primer: 5′-CATGTGGTCTCTCTTTTCGTTGGG-3′. The conditions for nested EGFP PCR were 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, for 10 cycles with the EGFP1 primers, using the RoboCycler 40 (Stratagene, La Jolla, CA). A 3-μl reaction mixture (from a total of 50 μl) was then used for the nested EGFP PCR with EGFP2 primers, under the same conditions but for 35 cycles. A fragment of 380 bp was expected and produced from control EGFP plasmid DNA.

TABLE 4. RETROVIRAL TRANSDUCTION OF CULTURED CELLS, USING THE SPINOCULATION AND/OR RETRONECTIN METHODS: EXPERIMENT 8

Days in culture[b]	Method of transduction[c]	CD34+[d]	EGFP+[a]			
			CD45+	CD34+	CD13/33+	CFC
2 + 3	M	92	0	0	0	0
	S	92	12.6	10.3	11.2	8.5 ± 1
	S + RN	92	18.8	17.2	17.4	ND
	RN	91	24.0	23.0	24.0	ND
	BSA	93	0	0	0	0
5 + 3	M	51	0	0	0	0
	S	51	11.7	5.9	8.2	6.5 ± 1
	S + RN	48	17.3	12.0	13.6	19.7 ± 6
	RN	50	18.5	15.0	15.9	27.0 ± 6
	BSA	47	0	0	0	0
7 + 3	M	44	0	0	0	0
	S	40	3.5	2.7	3.2	17.9 ± 3
	S + RN	28	15.4	11.4	10.8	61.6 ± 7
	RN	32	13.8	13.5	12.6	52.0 ± 9
	BSA	32	0	0	0	0

[a]The percentage of EGFP+CD45+, CD34+, and CD13/33+ cells was assessed by flow cytometry. Percentage of EGFP+ CFCs and total CFCs was determined by fluorescence microscopy; EGFP+ CFC-Mix, CFC-GM, and BFU-E were observed, but the data are not broken down by type of CFC.

[b]CB CD34+ cells were cultured under culture condition 2 for 2, 5, or 7 days, transduced, and cultured for an additional 3 days.

[c]M, mock spinoculation; S, spinoculation; RN, incubation on RetroNectin; BSA, incubation on bovine serum albumin.

[d]Percentage of total CD34+ cells per total cells.

a transduction strategy used in several previous studies (Cheng *et al.*, 1998; van Hennik *et al.*, 1998), versus 7 days (total duration) of culture with a third addition of retroviral supernatant on day 5. We did not observe a significant difference in the percentages of cultured CD45+ or CD34+ cells that were EGFP+ in the populations of cells cultured for 7 versus 4 days. However, the percentages of EGFP+ cells and CFCs recovered from the animals 4 weeks posttransplantation were somewhat increased in the mice transplanted with cells that had been cultured for 7 days (Table 6), as compared with 4 days. Both erythroid and myeloid (EGFP+ CFCs were recovered from mice transplanted with both 4 and 7 day-cultured cells (data not shown).

We purified human CD34+ cells from the BMs of two highly engrafted mice transplanted with 7 day-cultured cells; evaluation and purification of CD34+ cells from the BMs of the

TABLE 5. INCREASE IN EFFICIENCY OF TRANSDUCTION ON RETRONECTIN AFTER DAILY ADDITION OF VIRAL SUPERNATANT TO *EX VIVO* CULTURES OF CORD BLOOD CD34+ CELLS: EXPERIMENT 9

Days in culture[b]	Method of transductions[c]	CD34+[a]	EGFP+[a]				
			CD45+	CD34+	CFC-Mix	CFC-GM	BFU-E
0	0	97	0	0	0 3 ± 1	0 27 ± 6	0 29 ± 5
3	1	89	9	9	ND	ND	ND
4	2	76	35	35	ND	ND	ND
5	3	72	42	40	ND	ND	ND
6	4	64	49	51	ND	ND	ND
7	5[d]	54	54	52	8 ± 1 15 ± 1	73 ± 1 98 ± 6	48 ± 3 136 ± 7
8	5[d]	58	56	56	ND	ND	ND

[a]See Table 3 footnotes.

[b]CB CD34+ cells were cultured under culture condition 2 for 2 days, plated on RetroNectin, and cultured for an additional 6 days.

[c]Number of viral supernatant additions: each day from day 2 to day 6, cells were centrifuged and resuspended in fresh QBSF-60 + FKT and fresh viral supernatant (1:1).

[d]An aliquot of cells was left in culture from day 7 to day 8, without further additions of viral supernatant, to allow full expression of the EGFP transcript.

TABLE 6. COMPARISON OF THE EFFECT OF 4- VERSUS 7-DAY CULTURE ON THE EFFICIENCY OF TRANSDUCTION OF CD34$^+$ CELLS: EXPERIMENT 10

			EGFP^{+a}							
Days in culture[b]	*Number of transductions*[b]	*CD34^{+a}*	*CD45$^+$*	*CD34$^+$*	*CFC-Mix*	*CFC-GM*	*BFU-E*	*CD45$^+$ from chimera BM*	*CD34$^+$ from chimera BM*	*CFC from chimera BM*
4	2	99	38	35	45 ± 20	46 ± 10	18 ± 5	13 ± 2	ND	2 ± 1
7	3	39	44	36	48 ± 15	53 ± 13	28 ± 7	23 ± 2	19 ± 3	31 ± 8

[a]See Table 3 footnotes. In this experiment, we also transplanted cultured, transduced cells for measurement of EGFP$^+$SCID engrafting cells. On average, 1 (±1) × 10^6 human CD45$^+$ cells were recovered from the BMs of chimeras transplanted with day 4 cultured cells, while 4.8 (±6.7) × 10^6 human cells were recovered from the chimeras transplanted with day 7 cultured cells. This difference was not significant, considering the high variation in the levels of engraftment among the chimeras in this experiment.

[b]CB CD34$^+$ cells were cultured for 2 days, plated on RetroNectin and either cultured for an additional 2 days, with two additions of viral supernatant, or cultured for an additional 5 days with three additions of viral supernatant and replacement of the RetroNectin layer on day 5. In this experiment, we used retroviral supernatant collected in QBSF-60, to avoid the introduction of small amounts of FCS under condition 2. In preliminary studies, we had observed that comparable titers of viral supernatant are obtained when the packaging cell line is cultured overnight in QBSF-60, as compared with DMEM containing 10% FCS; viral supernatants obtained in either serum-free medium or DMEM + 10% FCS were equally efficient in transducing CB CD34$^+$ cells and CFCs (data not shown).

chimeras transplanted with day 4 cultured cells were not possible because of the low levels of engraftment in this group. We obtained ~10^6 viable CD34$^+$ cells from the BM of the two chimeras (pooled). Of these purified CD34$^+$ cells, 28% were EGFP$^+$ by flow cytometry (Fig. 4). After plating in CFC assays, 17% of the colonies generated by these CD34$^+$ cells purified from the BM of the chimeras were EGFP$^+$.

DISCUSSION

CB is currently in clinical use as an alternative source of HSCs for hematopoietic transplantation (Cairo and Wagner, 1997). Reports have demonstrated that it is possibe to genetically engineer CB HSCs (Lu *et al.*, 1993; Kohn *et al.*, 1995, 1998), but improved gene transduction protocols appear to be necessary to obtain therapeutic effects (Emerson, 1996; Kohn *et al.*, 1998). In the experiments discussed herein, we explored the effects of *ex vivo* suspension culture of CB CD34$^+$ cells on the ability of the cultured cell population to engraft *in vivo* and to express a retrovirally transduced gene.

Our first set of *ex vivo* cultures was performed with medium supplemented with 10% FBS plus six GFs (FL, KL, Tpo, IL-3, IL-6, and G-CSF). Previously, it had been shown that culture of human CB CD34$^+$CD38$^-$ cells with these GFs resulted in increases in numbers of total cells, progenitor cells, and LTC-ICs (Petzer *et al.*, 1996a,b). The same group also reported that cultured human CB CD34$^+$CD38$^-$ cells engrafted in NOD/SCID mice. They found that the frequency of SCID engrafting cells, by limiting dilution analysis, was approximately twofold higher in cultured cells then in fresh cells. However, these cells appeared to have reduced proliferative potential, since the numbers of progeny recovered from the BMs of the chimeras were somewhat decreased (Conneally *et al.*, 1997). Similarly, an independent report showed that the SCID repopulating capacity of CB CD34$^+$CD38$^-$ cells was slightly en-

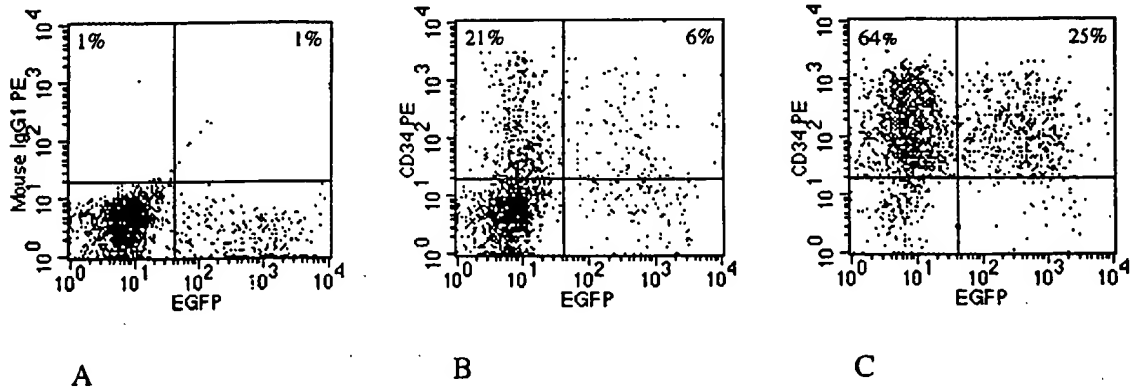

FIG. 4. Flow cytometric analysis of CD34$^+$ cells detected and isolated from the BMs of two mice transplanted with 7 day-cultured CB CD34$^+$ cells (from experiment 10, Table 6). (A) Isotype control; (B) cell population prior to the purification; (C) enriched CD34$^+$ cells after purification. The percentages of cells in selected quadrants are indicated.

hanced (fourfold) by *ex vivo* culture for 4 days in serum-free medium supplemented with the same GF cocktail, but was completely lost after 9 days of culture (Bhatia *et al.*, 1997), in spite of increases in CFC numbers. It was reported that when $CD34^+$ cells were cultured in serum-free medium in the presence of KL, FL, and either IL-3 or Epo/IL-6, the SCID repopulating capacity decreased by 2-fold after 1 day of culture and by 25-fold by day 3 (Rebel *et al.*, 1999). The results of our *in vitro* assays were consistent with these studies, in that culture of CB $CD34^+$ cells, in both the culture conditions tested, resulted in increases in numbers of total cells, $CD34^+$ cells, CFCs, and LTC-ICs. In our experiments, the SCID repopulating capacity (calculated as SEP)(Leung *et al.*, 1998) of cultured cells was 2.8-fold decreased, as compared with fresh cells. We had hypothesized that increased numbers of colony-forming progenitor cells (which generate *in vitro* colonies enumerated 2 weeks after plating) might correlate with higher levels of *in vivo* engraftment 2–3 weeks after transplant, but this was not observed. SEP was somewhat lower for cultured than for fresh cells, even at these time points early after transplantation. Overall in our experiments (averaged), 1 fresh CB $CD34^+$ cell yielded 38 $CD45^+$ cells in the BMs of the chimeras, while 1 initially cultured CB $CD34^+$ cell yielded only 14 $CD45^+$ cells. Thus, we did not obtain evidence for the hypothesis that *ex vivo* culture increases *in vivo* engrafting capacity. Nevertheless, these results confirm and extend the conclusion that a cultured CB $CD34^+$ cell population is capable of *in vivo* engraftment, with the capacity to generate high levels of multilineage hematopoiesis at both short-term and long-term time points after transplantation in the NOD/SCID chimera model. It is possible that slightly different culture conditions may further improve the SEP of the cultured cell population. For instance, several groups have shown that maximal CFC, LTC-IC, and SEP expansion occurs when subpopulations of $CD34^+$ cells are plated at low cell concentrations (Emerson, 1996; Conneally *et al.*, 1997), with continuous resupply of fresh medium and GFs (Emerson, 1996; Piacibello *et al.*, 1999), and with extended periods of culture (Piacibello *et al.*, 1999). These factors may be responsible for the variability in *ex vivo* "expansion" observed among different studies.

Several groups have reported that inclusion of IL-3 in *ex vivo* culture medium results in diminished stem/progenitor cell function (Yonemura *et al.*, 1996; Dao *et al.*, 1997; Zandstra *et al.*, 1997). Others found that *ex vivo* culture in FL plus Tpo, with or without KL, but without "late-acting" growth factors was sufficient to increase progenitor cell numbers (Piacibello *et al.*, 1997). Furthermore, it was shown (Luens *et al.*, 1998) that culture of PKH26-labeled BM $CD34^+Thy1^+Lin^-$ cells in FL, KL, plus Tpo for 6 days resulted in persistent $CD34^+$ cells, all of which had divided (i.e., had the phenotype $CD34^{high}PKH26^{low}$); nevertheless, the cultured, purified $CD34^+$ cells retained the capacity to engraft in immunodeficient mice. Therefore, we next investigated the effects of *ex vivo* culture in the presence of only these three GFs (FL, KL, and Tpo). To avoid unknown effects of variable serum proteins and to better enable future clinical application, we utilized a serum-free medium, QBSF-60. The effects of *ex vivo* culture in these simpler conditions were similar to those observed after culture in medium containing FBS plus six GFs: the cultured cells generated high levels of short-term and long-term human engraftment, although the SEP was slightly decreased as compared with fresh cells. In averaging the experiments conducted in culture condition 2, the ratio of SEP for cultured versus fresh cells was 0.9. It should be noted that the decrease in SEP of cultured versus fresh cells varied among experiments and did not always reach significance.

Taken together with the current literature, the above results suggested that culture of $CD34^+$ cells in serum-free medium containing FL, KL, plus Tpo might result in enhanced retroviral gene transduction of *in vivo* SCID engrafting cells. We decided to test this hypothesis by transducing cultured versus fresh $CD34^+$ cells with the MSCV-based MGIN vector carrying the EGFP reporter gene. Previous observations showed that transduction with MSCV-based vectors yields a higher level of transgene expression in hematopoietic progeny, as compared with cells transduced using the conventional Moloney murine leukemia virus (Mo-MuLV)(Cheng *et al.*, 1998; van Hennik *et al.*, 1998). Since there are several different methods of retroviral transduction, we began by testing different transduction approaches. We evaluated these approaches by *in vitro* assays of transduction efficiency (experiments 7–9). We then selected a single approach that worked well and tested this approach by both *in vivo* and *in vitro* assays (experiment 10). We first explored the effects of 2 days of *ex vivo* "preculture" on gene transduction; similar durations of preculture have been utilized widely to enhance retroviral transduction efficiency (Larochelle *et al.*, 1996; Cheng *et al.*, 1997, 1998; Dao *et al.*, 1997; Conneally *et al.*, 1998; Schilz *et al.*, 1998; van Hennik *et al.*, 1998). As expected, the level of EGFP expression in hematopoietic progenitor cells was increased severalfold by a 2-day *ex vivo* preculture (Table 3). It had been suggested that immature $CD34^+38^{low}$ cells may require at least 5 days of preculture in the presence of GF exposure before they divide (Conneally *et al.*, 1997, 1998). Therefore, we next explored the effects of different lengths of preculture (in condition 2) on gene transduction efficiency by either spinoculation, transduction in the presence of RetroNectin, or a combination of these two transduction methods. Transduction by spinoculation (alone) resulted in EGFP expression in up to 30% of CFCs. This result is consistent with previously published reports using murine retroviral vectors (Nolta *et al.*, 1996; Cheng *et al.*, 1997, 1998; Conneally *et al.*, 1998). When transduction was performed on RetroNectin, up to 60% of CFCs were $EGFP^+$. It is possible that the enhancement provided by the use of RetroNectin in this single experiment may be an overestimate, especially since toxicity due to the presence of Polybrene may have negatively affected transduction by spinoculation (Conneally *et al.*, 1998). In addition, the lengths of exposure of cells to the viral supernatant were different among the groups. Further experiments and an optimized spinoculation protocol (i.e., using protamine sulfate [Conneally *et al.*, 1998] instead of Polybrene) will be required to determine which procedure is more efficient in transducing HSCs. Nevertheless, both these experiments indicate that cells in this serum-free medium with three GFs can be readily transduced with a retroviral vector. Since only a fraction of the HSCs may divide during transduction on any single day and since the half-life of retrovirus is <1 day (Kotani *et al.*, 1994), we tested whether daily addition of fresh virus in the presence of RetroNectin might result in increasing levels of transduction. Intriguingly, up to 50% of the human cells in NOD/SCID mice carried the retroviral marker after transplantation of cells that

had undergone multiple transductions by spinoculation (Schilz *et al.*, 1998). Experiment 9 (Table 5) shows a limited increase in the percentage of cells that expressed EGFP after multiple additions of viral supernatant. The percentage of cells that expressed EGFP increased slightly, from ~40% (two transductions) to 56% (five transductions). RetroNectin was not replaced throughout the duration of this experiment, so it is possible that saturation of RetroNectin binding sites after the early additions of viral supernatant may have prevented greater increases in transduction efficiency.

Since Luens *et al.* (1998) had reported that $CD34^+$ cells, repurified after 6 days of culture in the above three GFs, had all divided and were capable of engraftment in the Hu/SCID model, we decided to test whether the retrovirally transduced cells in our cultures could engaft *in vivo* in NOD/SCID mice (experiment 10). As shown in Table 6, cells cultured and transduced for either 4 or 7 days were capable of engraftment in NOD/SCID mice, as assessed after 4 weeks. $EGFP^+$ CFCs were recovered from the chimeras, and $EGFP^+CD34^+$ cells purified from the chimeras were able to generate $EGFP^+$ hematopoietic colonies.

In summary, we have first confirmed that *ex vivo* cultured CB $CD34^+$ cells can generate multilineage engraftment in NOD/SCID mice. Whether modifications to these culture conditions will result in actual expansion of SEP will be the subject of further investigations. For example, future research may indicate that IL-6 or a fusion protein of the IL-6α chain joined to an IL-6 receptor fragment may increase the survival/proliferation of SCID repopulating cells (Rappold *et al.*, 1998). We have also shown that serum-free culture with FL, KL, and Tpo enhances the efficiency of gene transfer into $CD34^+$ progenitor cells, by *in vitro* and *in vivo* assays. In the experiments presented herein, cells precultured in serum-free medium with three GFs were efficiently transduced by one or more transductions with MSCV and RetroNectin. The cultured, transduced cells generated $EGFP^+$ human cells in NOD/SCID transplant recipients. Transduction may be further enhanced by improved retroviral vectors and packaging cell lines, e.g., MSCV vectors pseudotyped with the gibbon ape leukemia virus envelope protein (Kiem *et al.*, 1997; Schilz *et al.*, 1998; van Hennik *et al.*, 1998), or Mo-MuLV pseudotyped with the vesicular stomatitis virus G protein (Rebel *et al.*, 1999). We hypothesize that such strategies, when fully optimized with respect to vector, titer, and transduction conditions, will support adequate levels of gene transduction to provide therapeutic benefit in clinical settings. Testing for persistence of transducted cells for a longer times (>2–3 months) should be done in chimera models prior to such clinical studies.

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CULTURE OF ANIMAL CELLS

A Manual of Basic Technique

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Chapter 13 Characterization

INTRODUCTION

There are four main reasons for characterizing a cell line:

(1) Correlation with the tissue of origin: (a) identification of the lineage to which the cell belongs, (b) position of the cells within that lineage, i.e., the precursor or differentiated status, and (c) whether transformed or not.

(2) Monitoring for instability and variation (see Chapter 17).

(3) Checking for cross contamination (see Chapter 16) and confirmation of species of origin.

(4) Identifying selected sublines or hybrid cell lines requiring demonstration of unique features.

Species Identification

Chromosomal analysis (see below) is the best method of distinguishing between species. Isoenzyme electrophoresis is also a good diagnostic test and is quicker than chromosomal analysis, but requires the appropriate apparatus and reagents (see below). In practice a combination of the two is often used and will give unambiguous results [Hay, 1986].

Lineage or Tissue Markers

Cell surface antigens. These markers are particularly useful in sorting subspecies of lymphocytes, and have also been effective in discriminating epithelium from stroma with antibodies such as anti-EMA [Heyderman et al., 1979] and anti-HMFG 1 and 2 [Burchell et al., 1983] and neuroectodermally derived cells (anti-2AB5) [Dickson et al., 1983] from other germ layer derived cells.

Intermediate filament proteins. These are among the most widely used lineage or tissue markers [Ramackers et al., 1982]. Glial fibrillary acidic protein (GFAP) for astrocytes and desmin for muscle are the most specific, while cytokeratin will mark epithelial cells and mesothelium. Vimentin, though usually restricted to mesodermally derived cells *in vivo*, can appear in other cell types *in vitro*.

Differentiated products. Hemoglobin for erythrocytic cells, myosin or tropomyosin for muscle, melanin for melanocytes, and serum albumin for hepatocytes are among the best examples of specific cell type markers, but, like all differentiation markers, depend on the complete expression of the differentiated phenotype.

Enzymes. Three parameters are available in enzymic characterization: (1) the constitutive level (i.e., in the absence of inducers or repressors); (2) the response to inducers and repressors; and (3) isozymic differences (see below). Creatine kinase BB isozyme is characteristic of neuronal and neuroendocrine cells as is neurone specific enolase; lactic dehydrogenase is present in most tissues but in different isozymic forms, and a high level of tyrosine aminotransferase, inducible by dexamethasone, is generally regarded as specific to hepatocytes.

Special functions. Transport of inorganic ions and water is characteristic of some epithelia such that, grown as monolayers, they will produce "domes," blisters in the monolayer caused by transport of water from the medium to the underside of the monolayer. This is found in kidney epithelium and some secretory epithelia. Other specific functions which can be expressed *in vitro* include muscle contraction and depolarization of nerve cell membrane.

Regulation. Although differentiation is usually regarded as an irreversible process, the level of expression of many differentiated products is under the regulatory control of environmental influences such as hormones, matrix, and adjacent cells (see Chapter 14). Hence the measurement of specific lineage markers may require preincubation of the cells in, for example, a hormone such as hydrocortisone, or growth of the cells on collagen of the correct type. Maximum expression of both tyrosine aminotransferase in liver cells and glutamine synthetase in glia require prior induction with dexamethasone. Glutamine synthetase is also re-

pressed by glutamine, so glutamate should be substituted in the medium 48 hr before assay [DeMars, 1957].

Lineage fidelity. Although many of the markers described above have been claimed as lineage markers, they are more properly regarded as tissue or cell type markers, as they are often more characteristic of the function of the cell than its embryologic origin. Cytokeratins occur in mesothelium and kidney epithelium although both of these derive from the mesoderm. Neurone specific enolase and creatine kinase BB are expressed in neuroendocrine cells of the lung although these are now recognized to derive from the endoderm and not from neurectoderm as one might expect of neuroendocrine type cells.

Unique Markers

These include specific chromosomal aberrations, enzymic deficiencies, isozymes, and drug resistance.

Transformation

This will be dealt with in Chapter 15.

MORPHOLOGY

This is the simplest and most direct technique used to identify cells. It has, however, certain shortcomings which should be recognized. Most of these are related to the plasticity of cellular morphology in response to different culture conditions, e.g., epithelial cells growing in the center of a confluent sheet are usually regular, polygonal, and with a clearly defined edge, while the same cells growing at the edge of a patch may be more irregular, distended, and if transformed, may break away from the patch and become fibroblastoid in shape. Subconfluent fibroblasts from hamster kidney or human lung or skin assume multipolar or bipolar shapes and are well spread on the culture surface, but at confluence they are bipolar and less well spread. They also form characteristic parallel arrays and whorls which are visible to the naked eye. Mouse 3T3 cells and human glial cells grow like multipolar fibroblasts at low cell density but become epithelial-like at confluence (Fig. 13.1). Alterations in the substrate [Gospodarowicz, 1978; Freshney, 1980], and the constitution of the medium [Coon and Cahn, 1966] can also affect cellular morphology. Hence, comparative observations should always be made at the same stage of growth and cell density in the same medium, and growing on the same substrate (see Fig. 13.1).

The terms "fibroblastic" and "epithelial" are used rather loosely in tissue culture and often describe the appearance rather than the origin of the cells. Thus a bipolar or multipolar migratory cell, the length of which is usually more than twice its width, would be called "fibroblastic," while a monolayer cell which is polygonal, with more regular dimensions, and which grows in a discrete patch along with other cells, is usually regarded as "epithelial." However, where the identity of the cells has not been confirmed, the terms "fibroblast-like" or "fibroblastoid" and "epithelial-like" or "epithelioid" should be used.

Frequent brief observations of living cultures, preferably with phase-contrast optics, are more valuable than infrequent stained preparations studied at length. They will give a more general impression of the cell's morphology and its plasticity and will also reveal differences in granularity and vacuolation which bear on the health of the culture. Unhealthy cells often become granular and then display vacuolation around the nucleus (see Fig. 10.1).

It is useful to keep a set of photographs for each cell line as a record in case a morphological change is suspected. This record can be supplemented with photographs of stained preparations.

Staining

A polychromatic blood stain, such as Giemsa, provides a convenient method of preparing a stained culture. The recommended procedure is as follows.

Outline

Fix the culture in methanol and stain directly with Giemsa. Wash and examine wet.

Materials

BSS
undiluted Giemsa stain
methanol
deionized water

Protocol

1.
Remove medium and discard.
2.
Rinse monolayer with BSS, and discard rinse.
3.
Add BSS:methanol, 1:1, 5 ml per 25 cm^2.
4.
Discard 50% methanol/BSS mixture and replace with fresh methanol. Leave for 10 min.
5.
Discard methanol and replace with fresh anhy-

Chapter 14
Induction of Differentiation

As discussed in Chapter 2, when cells are cultured and propagated as a cell line, the resultant cell phenotype is often different from the characteristics predominating in the tissue from which it was derived. This is due to several factors, many of them as yet undefined, which regulate the geometry, growth, and function *in vivo* but which are absent from the tissue culture environment. Before considering these systematically it is first necessary to state what is meant by *differentiation* in this context. The term will be used to define the process leading to the expression of phenotypic properties characteristic of the functionally mature cell *in vivo*. It does not imply that the process is complete or that it is irreversible. There will be processes such as the cessation of DNA synthesis in the erythroblast nucleus which are not normally reversible, but, for simplicity, no attempt will be made to distinguish these from reversible processes such as the induction of albumin synthesis in hepatocytes which is lost under certain circumstances but may be reinduced.

Differentiation will be used to describe the combination of constitutive and adaptive properties found in the mature cell. *Commitment*, on the other hand, will imply an irreversible progression from a stem cell to a particular defined lineage endowing the cell with the potential to express a limited repertoire of properties either constitutively or when induced to do so.

Terminal differentiation implies that a cell has progressed down a particular lineage to a point at which the mature phenotype is fully expressed and beyond which the cell cannot progress. In principle this need not exclude cells which can revert to a less differentiated phenotype and resume proliferation, such as a fibrocyte, but in practice the term tends to be reserved for cells like neurons, skeletal muscle, or keratinized squames where differentiation is irreversible.

Dedifferentiation has been used to describe the loss of the differentiated properties of a tissue when it becomes malignant or when it is grown in culture. As these are complex processes with several contributory factors including cell death, selective overgrowth, adaptive responses, and loss of certain phenotypic properties, the term should be used with great caution, or not at all. When used correctly dedifferentiation means the loss by a cell of the specific phenotypic properties associated with the mature cell. When it occurs, this is most probably an adaptive process implying that the differentiated phenotype may be regained given the right inducers (see also Chapter 2).

STAGES OF COMMITMENT AND DIFFERENTIATION

There are two main pathways to differentiation in the adult organism (Fig. 14.1). Typically a small population of totipotent or pluripotent undifferentiated stem cells gives rise to committed precursor cells which will progress towards terminal differentiation, losing their capacity to divide as they reach the terminal stages. This gives rise to a fully mature, differentiated cell which will normally not divide. Alternatively, cells such as fibrocytes may respond to a local reduction in cell density and/or the presence of one or more growth factors by losing some of their differentiated properties, e.g., collagen synthesis, and re-entering the cell cycle. When the tissue has regained the appropriate cell density by division, cell proliferation stops and differentiation is reinduced.

The first process is used where continual renewal is required, such as in the hemopoietic system, the skin, and the gastric mucosa, while the second is used where regeneration is not continual and requires quick mounting in response to trauma, such as in wound repair or liver regeneration. In the first, amplification is possible by having several cell divisions at each precursor stage but takes longer than the rapid recruitment of a higher proportion of the total cell population into a limited number of divisions available in the second.

PROLIFERATION AND DIFFERENTIATION

As differentiation progresses, cell division is reduced and eventually lost. In most cell systems cell proliferation is incompatible with the expression of

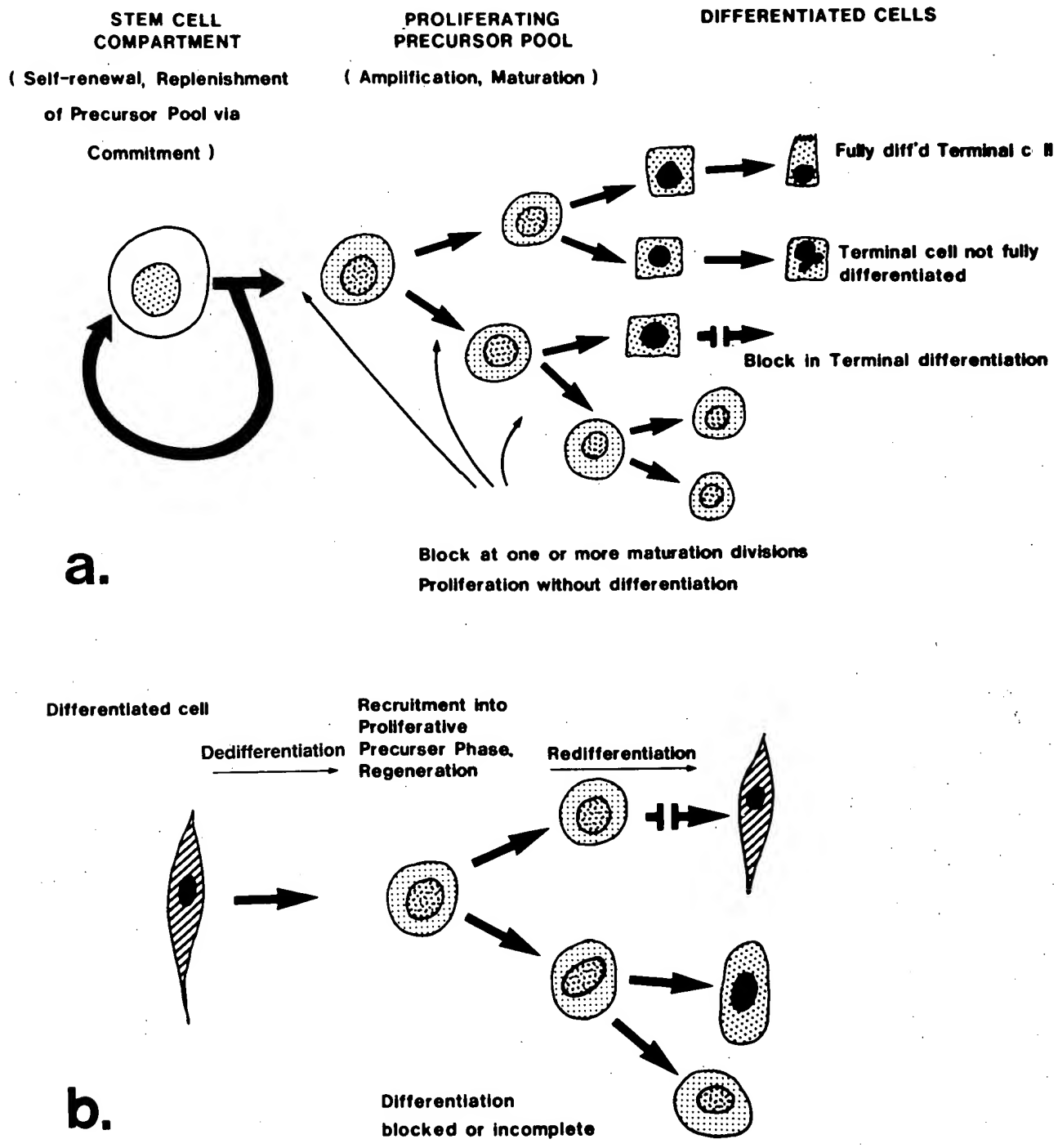

Fig. 14.1. *Alternative pathways for cell differentiation. a. Generation from stem cell b. Generation by recruitment from differentiated cells. The blocks refer to points at which expression of differentiation may be inhibited in vitro, but could apply equally to blocks found in neoplasia. (Reproduced from Freshney, 1985, by permission of the publisher).*

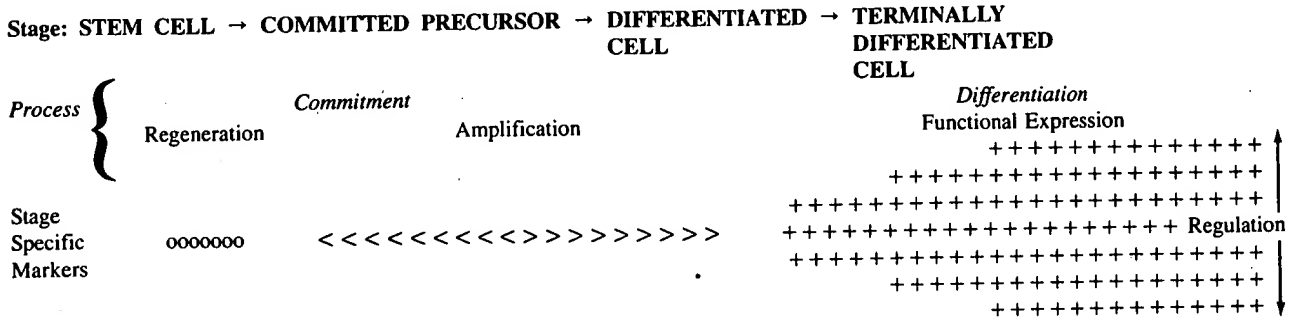

Fig. 14.2. *Stages in cell differentiation. Stem cells are capable of regeneration without commitment or commitment division leading to a precursor cell which is still capable of cell proliferation and hence amplification of the lineage. Successive divisions lead to further differentiation, in the presence of the appropriate inducer environment, until, finally, a stage may be reached where no further division is possible. This is terminal differentiation. Phenotypic expression may be regulated quantitatively at terminal differentiation from the constitutive to the induced state by the action of hormones, metabolites, etc.*

differentiated properties. Tumor cells can sometimes break this restriction, and in melanoma, for example, melanin continues to be synthesized while the cells are proliferating. Even in these cases, however, synthesis of the differentiated product increases when division stops.

There are severe implications for this relationship in culture, where the major objective for many years has been the propagation of cell lines and the production of large numbers of cells for biochemical or molecular analysis. Where the incompatibility of differentiation and proliferation is maintained, it is not surprising to find that the majority of cell lines do not express fully differentiated properties.

This fact was noted many years ago by the exponents of organ culture (see Chapter 22), who set out to retain three-dimensional, high cell density tissue architecture and prevent dissociation and selective overgrowth of undifferentiated cells. However, although of considerable value in elucidating cellular interactions regulating differentiation, organ culture has always suffered from the inability to propagate large numbers of identical cultures, particularly if large numbers of cells are required, and the heterogeneity of the sample, assumed to be essential for the maintenance of the tissue phenotye has, in itself, made the ultimate biochemical analysis of pure cell populations, and their responses, extremely difficult.

Hence, in recent years, there have been many attempts to reinduce the differentiated phenotype in pure populations of cells by recreating the correct environment, and, by doing so, defining individual influences exerted on the induction and maintenance of differentiation. This usually implies cessation of cell division and creation of an interactive high-density cell population as in histotypic or organotypic culture. This will be discussed in greater detail below.

COMMITMENT AND LINEAGE

Progression from a stem cell to a particular pathway of differentiation usually implies a rapid increase in commitment with advancing stages of progression (see Figs. 2.3 and 14.2). A hemopoietic stem cell after commitment to lymphocytic differentiation will not change lineage at a later stage and adopt myeloid or erythrocytic characteristics. Similarly, a primitive neurectodermal stem cell, once committed to become a neurone, will not change to a glial cell. This is not to say that if the inductive environment is altered early enough (before commitment) that a cell can alter its destiny or even adopt a mixed phenotype under artefactual or pathological circumstances.

Commitment may therefore be regarded as the point, between the stem cell and a particular precursor stage, where a cell or its progeny can no longer transfer to a separate lineage.

Many claims have been made in the past for cells transferring from one lineage to another. Perhaps the best substantiated of these is the regeneration of the amphibian lens by recruitment of cells from the iris [Clayton et al., 1980; Cioni et al., 1986]. Since the iris can be fully differentiated and still regenerate lens,

this has been proposed as proper transdifferentiation. It is, however, one of the few examples, and most other claims have been from tumor cell systems where the origin of the tumor population may not be clear. Small cell carcinoma of the lung has been found to alter to squamous carcinoma following recurrence after relapse from chemotherapy. Whether this implies that one cell type, the Kulchitsky cell [de Leij et al., 1985], presumed to give rise to small cell lung carcinoma, changed its commitment or whether the tumor originally derived from a multipotent stem cell and on recurrence progresses down a different route is still not clear [Gazdar et al., 1983; Goodwin et al., 1983; Terasaki et al., 1984]. Similarly, the K562 cell line was isolated from a myeloid leukemia, but subsequently was shown capable of erythroid differentiation [Andersson et al., 1979b]. Rather than a committed myeloid precursor converting to erythroid, it seems more likely that the tumor arose in the common stem cell known to give rise to both erythroid and myeloid lineages. For some reason, as yet unknown, continued culture favoured erythroid differentiation rather than the myeloid features seen in the original tumor and early culture.

In some cases, again in cultures derived from tumors, a mixed phenotype may be generated. The C_6 glioma of rat expresses both astrocytic and oligodendrocytic features and these may be demonstrated simultaneously in the same cells.

In general, however, these cases are unusual and restricted to tumor cultures. Most cultures from normal tissues, although they may differentiate in different directions, once committed will not alter to a different lineage. This raises the question of the actual status of cell lines derived from normal tissues. This has been dealt with already in Chapter 2 and the conclusion reached that most cultures are derived from: (1) stem cells which may differentiate in one or more different directions, e.g., lung mucosa which can become squamous or mucin-secreting depending on the stimuli; (2) committed precursor cells which will stay true to lineage; or (3) differentiated cells such as fibrocytes which may dedifferentiate and proliferate, but still retain lineage fidelity. Some mouse embryo cultures, loosely called fibroblasts, probably more correctly belong to (1) as they can be induced to become adipocytes, muscle cells, and endothelium as well as fibrocytes.

Cell lines may have different degrees of commitment depending on the "stemness" or precursor status of the cells from which they were derived; but unless the correct environmental conditions are re-established and proliferation is encouraged they will remain at the same position in the lineage.

MARKERS OF DIFFERENTIATION

Before studies of differentiation, its properties, and the regulation of its expression can be made, marker properties must be defined which will allow differentiation to be recognized. Markers expressed early and retained throughout subsequent maturation stages are generally regarded as lineage markers, e.g., intermediate filament proteins such as the cytokeratins (epithelium) [Moll et al., 1982] or glial fibrillary acidic protein (astrocytes) [Bignami et al., 1980; Eng and Bigbee, 1979]. Markers of the mature phenotype representing terminal differentiation are more usually specific cell products or enzymes involved in the synthesis of these products, e.g., hemoglobin in an erythrocyte, serum albumin in a hepatocyte, transglutaminase in a differentiating squame [Schmidt et al., 1985], or glycerol phosphate dehydrogenase in an oligodendrocyte [Breen and De Vellis, 1974] (see Table 2.2). These properties are often expressed well after commitment and are more likely to be reversible and under adaptive control by hormones, etc.

Differentiation should be regarded as the expression of one or preferably more than one of these marker properties. While lineage markers are helpful in confirming cell identity, the expression of the functional properties of the mature cells is the best criterion for terminal differentiation.

INDUCTION OF DIFFERENTIATION

There are four main parameters governing the control of differentiation and these are summarised in Figure 14.3.

Soluble Inducers

(Table 14.1) These include established endocrine hormones such as hydrocortisone, glucagon, and thyroxine (or triiodotyrosine), paracrine factors released by one cell and influencing adjacent cells, which as yet are poorly characterized (e.g., TGFβ from platelets, prostaglandins, NGF, glia maturation factor) [Lim and Mitsunobu, 1975], alveolar maturation factor [Post et al., 1984], vitamins such as vitamin D_3 and retinoic acid (see Table 14.1), and inorganic ions, particularly Ca^{2+}, where high Ca^{2+} promotes keratinocyte differentiation, for example (see Chapter 20).

coverslip, and examine by incident light 488 nm excitation.

Analysis. Cells fluorescing green are viable, those fluorescing red only, non-viable. Express viability as percentage fluorescing green as a proportion of the total.

Chromium release. Reduced $^{51}Cr^{3+}$ is taken up by viable cells and oxidized to $^{51}Cr^{2+}$ to which the membrane of viable cells is impermeable [Holden et al., 1973; Zawydiwski and Duncan, 1978]. Dead cells release the $^{51}Cr^{2+}$ into the medium. A reduction in viability is detected by γ-counting aliquots of medium from cultures labeled previously with $Na_2{}^{51}CrO_4$ for released ^{51}Cr. The test works well for a few hours but over longer periods spontaneous release of ^{51}Cr may be a problem.

Analysis. Express counts released as a percentage of total (medium + cells) and plot against time.

This method allows comparison of different toxic stimuli but does not give an absolute figure for percentage viable cells. It is often used to measure cytotoxic T-lymphocyte activity.

Metabolic tests. Alterations in glycolysis and respiration [Dickson and Suzangar, 1976] enzyme activity [DiPaulo, 1965], and incorporation of labeled precursors [Freshney, Paul, and Kane, 1975] have all been used to measure response to potentially toxic stimuli. Although these are often interpreted as viability or survival assays, they are not and should be interpreted solely as metabolic responses, specific to the parameter measured. Application to cell survival is limited, but comparison of relative population responses is possible if culture is continued after removal of drug for two to three population doublings (see below).

Protocols for measuring precursor uptake and total protein or DNA are given in Chapter 18.

Long-Term Tests—Survival

While short-term tests are convenient and usually quick and easy to perform, they only reveal cells which are dead (i.e., permeable) at the time of the assay. Frequently, cells subjected to toxic influences, e.g., antineoplastic drugs, will only show an effect several hours or even days later. The nature of the tests required to measure viability in these cases is necessarily different since, by the time the measurement is made, the dead cells may have disappeared. Long-term tests are often used to demonstrate the metabolic or proliferative capacity of cells *after* rather than during exposure to a toxic influence. The objective is to measure survival rather than short-term toxicity, which may be reversible.

The ability of cells to survive a toxic insult has been the basis of most cytotoxicity assays. In this context survival implies the retention of regenerative capacity and is usually measured by plating efficiency as would be the case with bacteria or other microorganisms. Unfortunately, many animal cells have poor plating efficiencies, particularly normal cells, freshly isolated, so a number of alternatives have been devised for assaying cells at higher densities, e.g., in microtitration plates. None of these tests measures survival directly. Instead the net increase in cell number (growth curve), the increase in total protein or DNA, or the residual ability to synthesize protein or DNA is determined. "Survival" in these cases is defined as the retention of metabolic or proliferative ability by the cell population as a whole; such assays cannot discriminate between a reduction in metabolic or proliferative activity per cell and a reduced number of cells.

Plating efficiency, as described in Chapter 18, is the best measure of survival and proliferative capacity, provided that the cells plate with a high enough efficiency that the colonies can be considered representative. Though not ideal, anything over 10% is usually acceptable.

Since the colony number may fall at high toxic concentrations, it is usual to compensate by seeding more cells so that approximately the same number of colonies form at each concentration. This removes the risk of cell concentration influencing survival and improves statistical reliability. In addition cells should be plated on a preformed feeder layer the density of which ($5 \times 10^3/cm^2$) greatly exceeds that of the cloning cells, where the plating efficiencies of controls is $< 100\%$.

A typical survival curve is prepared as follows:

Outline

Treat cells with experimental agent at a range of concentrations for 24 h. Trypsinize, seed at low cell density, and incubate for 1–3 wk. Stain and count colonies.

Materials

25 cm^2 flasks
6- or 9-cm petri dishes
PBSA
0.25% trypsin
growth medium
hemocytometer or cell counter

Defining Optimum Conditions for the Ex Vivo Expansion of Human Umbilical Cord Blood Cells. Influences of Progenitor Enrichment, Interference with Feeder Layers, Early-Acting Cytokines and Agitation of Culture Vessels

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ABSTRACT

Ex vivo expansion of human umbilical cord blood cells (HUCBC) is explored by several investigators to enhance the repopulating potential of HUCBC.

We performed experiments using either Ficoll-separated or CD34$^+$-selected HUCBC from the same donation in serum-free medium. CD34-purified HUCBC were cultured on either human umbilical vein endothelial cells (HUVEC) or irradiated bone marrow-derived stroma cells (BMSC) with addition of different cytokines. In addition, we tested the expansion of HUCBC in culture vessels with continuous rotation.

CD34 enrichment led to a significant increase in the expansion factor of CD34$^+$ cells compared with unmanipulated HUCBC. BMSC were more efficient in amplifying early progenitors than HUVEC. Optimum results were reached by a combination of SCF, FLT-3L at 300 ng/ml and IL-3 at 50 ng/ml. No significant improvement in the expansion of CD34$^+$/38$^-$ primitive progenitors could be obtained with other combinations. Addition of megakaryocyte-derived growth and development factor to each growth factor cocktail improved the expansion results. Continuous rotation of culture vessels did not ameliorate the expansion rate of the analyzed subsets. Culture conditions separating stroma and HUCBC by a semipermeable membrane improved the expansion factors of CD34$^+$, CD34$^+$/38$^-$, and CD34$^+$/41$^+$ cells and CFU-GM compared with contact cultures. These data might be useful when designing culture systems for clinical scale ex vivo expansion of HUCBC. *Stem Cells 1999;17:19-24*

INTRODUCTION

Human umbilical cord blood cells (HUCBC) have become an attractive source of hematopoietic precursors for allogeneic blood stem cell transplantation in children with inborn errors or malignant diseases [1, 2]. One major advantage of HUCBC in comparison with peripheral blood stem cells (PBSC) or bone marrow (BM) might be the reduced incidence of acute graft-versus-host disease caused by cord blood grafts [3]. Although HUCBC are easy to collect and store, the introduction into transplantation protocols for adults has been hampered by the limited number of progenitors contained in one cord blood harvest. The prolonged time to engraftment as well as the existence of very early hematopoietic progenitors have led hematologists to explore ex vivo expansion of HUCBC for clinical use [4].

Optimal consistence of different media as well as different growth factor cocktails have been evaluated in detail [5, 6]. Those experiments resulted in efficient expansion of progenitors, but long-term culture initiating cells (LTC-IC) or mouse-repopulating cells have not been amplified convincingly thus far. There are several studies indicating the importance of medium changes or perfusion

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of culture vessels as well as stirred conditions for enhanced expansion rates [7, 8]. Clinical scale bioreactors have been designed and tested in phase I studies in patients receiving expanded BM or PBSC after nonmyeloablative conditioning therapies [9].

Intermittent interactions of HUCBC with stromal cells, elimination of metabolites from the culture medium, oxygenation and pH have been shown to be independent factors which must be controlled in bioreactor systems [10]. In a series of experiments, we tried to define optimal conditions for culture of HUCBC in static culture conditions. We tested the influences of CD34 enrichment, different feeder layers, and megakaryocyte-derived growth and development factor (MGDF) on cell expansion kinetics. Additional experiments were performed to investigate the role of physical stromal contact by introduction of semipermeable membranes into culture dishes. Effects of continuous rotation of culture vessels were evaluated and compared with those of static cultures.

Materials and Methods

Cell Separation

Cord blood was collected and diluted with phosphate-buffered saline (PBS) Dulbecco's without magnesium and calcium, pH 7.2 (GIBCO; Paisley, Scotland). Density gradient centrifugation was performed as follows: cells were centrifuged with Immuflot (Immucor GmbH; Rödermark, Germany) at 800 *g* for 20 min, mononuclear cells were collected and washed twice with PBS, supplemented with 0.5% human serum albumin ([HSA] 5%, Immuno GmbH; Heidelberg, Germany). Pellets were resuspended in 5 ml of CellGro® SCGM ([stem cell growth medium], Boehringer Ingelheim Bioproducts; Mannheim, Germany). Cells were counted automatically by Technicon H 3 RTC™ (Bayer Diagnostics GmbH; München, Germany).

In some cases, lysis of red cells in HUCB was tested. About 50 ml of cord blood were mixed with 200 ml of ice-cold lysis-buffer, consisting of 8.29 g ammonium chloride, 1.00 g potassium hydrogen carbonate, and 0.037 g sodium-EDTA in one liter aqua with a pH of 7.4. After 15 min of incubation, the nucleated cells were separated by centrifugation, supernatant was removed, and the pellet was resuspended in 5 ml of CellGro® SCGM (Boehringer Ingelheim).

Establishment of Stromal Layers

Bone marrow aspirates were obtained from healthy donors after informed consent. The marrow was mixed with the same volume of PBS, and mononuclear cells were separated by density gradient centrifugation. Washed cells were resuspended in stroma medium (RPMI 1640, Dutch Modification, supplemented with 10% fetal calf serum [FCS], 2 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 1.0 µmol/l hydrocortisone).

5×10^6 cells were placed in 80-cm² flasks (Nunc; Wiesbaden, Germany) and maintained at 37°C and 5% CO_2. Half the medium was exchanged twice a week. After two weeks, confluent adherent layers were passaged with trypsin-EDTA solution 1X (Sigma-Aldrich; Steinheim, Germany). Trypsin was quickly inactivated with FCS-containing medium and centrifuged at 200 *g* for 10 min. The pellet was resuspended in stromal medium, and the cells of one 80-cm² culture vessel were transferred in six 25-cm² flasks or six six-well plates for further expansion experiments. Confluent layers were irradiated with 15 Gy before initiating culture experiments.

Establishment of HUVEC

Umbilical cords were placed in a PBS solution containing 10 mg/ml gentamycin, 250 µg/ml amphotericin B, and 5,000 U/ml sodium-heparin (Sigma-Aldrich). The umbilical cord vein was punctured and flushed with PBS several times. Collagenase solution 0.05% (Sigma-Aldrich) was instilled and incubated for 4 min at 37°C. To stop the enzymatic reaction, trypsin inhibitor solution from soybean was used. Residual cells were harvested by flushing the vein with PBS again.

An additional centrifugation step and resuspension of the cells in endothelial cell growth medium ([ECGM], PromoCell; Heidelberg, Germany) with a low content of FCS followed. HUVEC were placed in a culture flask coated with fibronectin (Sigma; 20 µg/ml) and maintained at 37°C and 5% CO_2.

Half the medium was exchanged three times a week until confluence was reached. Treatment with trypsin and further maintenance of the cultures was performed as mentioned above.

CD 34⁺ Progenitor Cell Purification

HUCBC were enriched using the MACS system (CD34 Selection Kit, Miltenyi Biotec GmbH; Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Briefly, HUCBC were washed and resuspended in PBS, 0.5% bovine serum albumin, and 5 mmol/l EDTA. Cells were first incubated with QBEND-10 antibody (mouse antihuman CD34) in the presence of human IgG as blocking reagent. After one cell wash, another incubation step with 100 µl MACS microbeads per 10^8 cells followed. Labeled cells were loaded onto a column installed in a magnetic field. Trapped cells were eluted after removal of the column from the magnet.

Methylcellulose Progenitor Culture

As described, 1×10^5 HUCBC or 1×10^3 CD34-enriched HUCBC were plated in a complete methylcellulose medium containing IMDM with 30% fetal bovine serum, 3 U/ml

erythropoietin, 50 ng/ml SCF, 20 ng/ml GM-CSF, 20 ng/ml IL-3, 20 ng/ml IL-6, and 20 ng/ml G-CSF (Methocult GF H4435, Stem Cell Technologies; Vancouver, Canada). Cultures were incubated at 37°C and 5% CO_2. The cultures were assessed at days 12 to 14 for the presence of burst-forming unit-erythroid, colony-forming unit-granulocyte-macrophage, and mixed colony-forming unit.

Expansion Cultures

HUCBC were cultured in 25 cm^2 flasks containing 10 ml serum-free medium (CellGro® SCGM, Boehringer Ingelheim) at 37°C and 5% CO_2 for eight days. Preformed stroma or HUVEC were used as feeder-layer, when indicated. Growth factor cocktail 1 contained SCF 300 ng/ml (Amgen; München, Germany), FLT3-ligand 300 ng/ml, and IL-3 50 ng/ml (Genzyme; Mannheim, Germany). Cocktail 2 contained the cytokines of cocktail 1 supplemented with G-CSF 450 ng/l (Amgen), IL-6 100 ng/ml (Genzyme), and erythropoietin 1 U/ml (Genzyme). MGDF (Amgen) was added at concentrations of 25 and 100 ng/ml. Unselected and CD34-enriched HUCBC were cultured at 5×10^5 and 1×10^4/ml, respectively.

Flow Cytometric Analysis

Analysis of the CD34 content of all samples before and after expansion was performed with FACS SCAN (Becton Dickinson; San Jose, CA) using a class III CD34 antibody HPCA-2-PE, (Becton Dickinson) and standard software LYSIS II. Double-color staining and analysis were performed for all samples using the following antibodies: anti-CD41-FITC, anti-CD38-FITC (Coulter-Immunotech Diagnostics; Miami, FL), and anti-CD117-PE (c-kit) (Dako Diagnostika; Glostrup, Denmark). Double-staining of CD34/CD38, CD34/CD117, and CD34/CD41 was performed to quantitate progenitor subsets.

To test for viability, propidium iodide (PI) staining was performed after all cultures. Annexin was used to determine the percentage of apoptotic cells. PI^-/$Annexin^+$ events defined early apoptosis, whereas PI^+/$Annexin^+$ signals were counted as late apoptosis.

Transwell Cultures

To test the influence of stroma contact, six-well plates (Nunc) were used. The culture dishes were separated by microporous membranes (0.2 or 3 µm) which do not allow cell migration but do allow diffusion of soluble factors like chemokines.

Rotating Cultures

A rotating 50 ml "3D Culture Module" (Heraeus; Osterode, Germany) was compared with static cultures. Culture vessels were maintained at 5% CO_2 and 37°C with a continuous rotation of 2 Upm.

Statistical Analysis

For most parameters, median and range are provided. Experiments evaluating different conditions were compared using the Wilcoxon-signed rank test for paired samples. Statistical significance was assumed when the two-tailed p value was below 0.05.

Results

CD34 Enrichment

CD34 enrichment and low-density culture showed significant enhancement of the expansion factors. Figure 1 compares the expansion factors of $CD34^+$ cells with and without prior immunomagnetic enrichment. The median expansion factor of $CD34^+$ cells was 4.2 (2.0-8.7) in unselected HUCBC and 29 (15-53) in the CD34-enriched controls ($p = 0.03$). Interestingly, the experiment with HUCBC at a purity of 96% did not provide the best results. The median expansion factor of total nucleated cells was 1.82 (0.8-3.2) for unselected samples compared with 237 (135-1,345) for CD34-enriched HUCBC ($p = 0.016$).

Feeder Layer

CD34 enriched HUCBC were either cultured on HUVEC ($n = 5$) or bone marrow-derived stroma ($n = 8$). Figure 2 depicts the median counts of $CD34^+$, $CD34^+/38^-$, and $CD34^+/41^+$ progenitors after culture either with or without a feeder layer. Even though a trend toward improved expansion could be measured after culture on HUVEC, the differences in cell numbers did not reach statistical significance for total nucleated cells (NC), $CD34^+$, and $CD34^+/38^-$ subsets ($p = 0.06$). In contrast, cultures on irradiated stroma led to significant increases in absolute

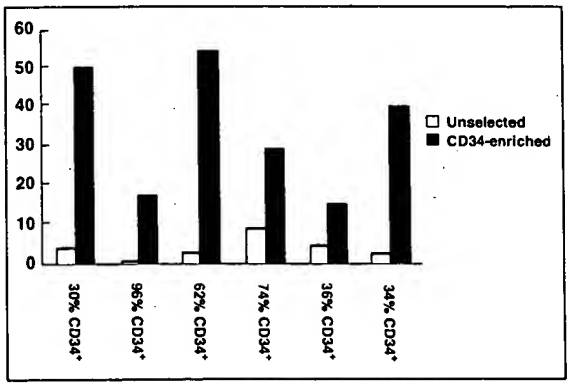

Figure 1. The bars represent the expansion factors reached in six experiments comparing either CD34 enriched or unselected HUCBC of the same origin. CD34 purity of each starting sample is provided on the x-axis.

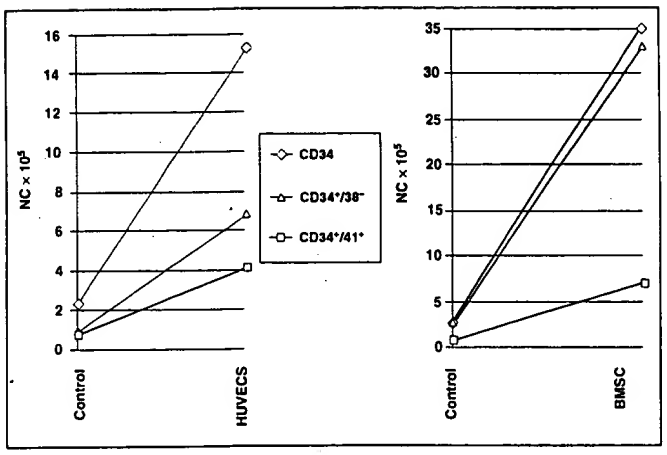

Figure 2. Influence of feeder layers. The starting cell concentration of CD34-enriched HUCBC was 1 x 10⁴/ml. Median absolute cell counts in 10 ml cultures after 8 days were compared to controls without feeder layer support. The cytokines used in these experiments were SCF and FLT-3L at 300 ng/ml and IL-3 at 50 ng/ml, respectively. HUVECS = human umbilical vein endothelial cells. BMSC = bone marrow-derived stroma cells; NC = nucleated cells.

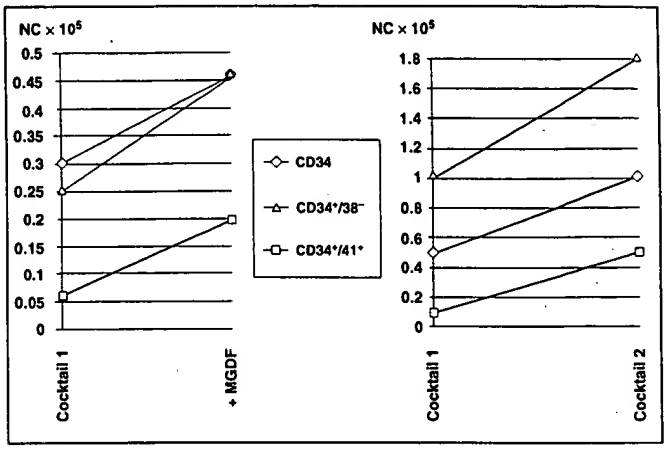

Figure 3. MGDF and other growth factors. A starting cell concentration of 1 x 10⁴/ml CD34 enriched HUCBC was inocculated. FLT-3L, SCF at 300 ng/ml and IL-3 at 50 ng/ml (cocktail 1) were either supplemented by MGDF at 25 ng/ml (left graph) or with G-CSF 450 ng/ml, EPO 1 U/ml and IL-6 at 100 ng/ml (right graph). The absolute numbers of CD34⁺, CD34⁺/38⁻ and CD34⁺/41⁺ cells after 8 days of culture are compared.

numbers of $CD34^+$ ($p = 0.008$), $CD34^+/38^-$, and $CD34^+/41^+$ subsets ($p = 0.016$).

MGDF and Other Growth Factors

A significant increase ($p = 0.0156$) of all $CD34^+$ cellular subsets was observed in cultures provided with 25 ng/ml MGDF. Nucleated cell counts did not differ significantly. Two growth factor cocktails containing either 300 ng/ml SCF, 300 ng/ml FLT-3L, 50 ng/ml IL-3 (cocktail 1), or cocktail 1 + 100 ng/ml IL-6, 450 ng/ml G-CSF, 1 U/ml Epo (cocktail 2) were compared (Fig. 3). Cocktail 2 showed better expansion results for total NC and $CD34^+$ cells ($p = 0.003$). Interestingly, the absolute increase of $CD34^+/38^-$ and $CD34^+/41^+$ early progenitors did not differ significantly ($p = 0.19$ and 0.06).

Viability and Apoptosis

Viability of HUCBC after eight days of culture varied between 89% and 94%. The median percentage of apoptotic cells after culture on BMSC was 8.1% ($r = 0.5$-23.5) compared with 14.9% ($r = 2.3$-25.8 in seven control experiments ($p = 0.21$).

Rotation

No significant differences in the expansion factors of $CD34^+$ subsets and CFU-GM could be found in cultures with continuous rotation at 2 Upm in comparison with static control cultures.

Stroma Contact

Table 1 shows the results of cultures comparing cultures in transwell experiments. The numbers provided are the expansion factors for each subset, respectively.

Table 1. Comparison of median expansion factors in cultures allowing no physical interference between stroma layers and HUCBC by means of a microporous membrane

	Contact	No-contact (2 µm)	*p*
Total nucleated cells	79 (45-88)	173 (138-210)	0.03
$CD34^+$	13 (8.4-17)	25.5 (21.1-28.8)	0.03
$CD34^+/38^-$	18.5 (10-62)	33 (28.9-88.4)	0.03
$CD34^+/41^+$	23.2 (10.6-61.4)	44.6 (39-80.1)	0.03
$CD34^+/117^+$	2.7 (1.17-9.8)	4.4 (2.2-21.4)	0.05
CFU-GM	2.8 (1.2-3.2)	7.3 (3.8-10.4)	0.03

The ranges are provided in parentheses.

Discussion

The results of static cultures obtained in our experiments are largely in the range published previously [11, 12]. In contrast with some reports, we found CD34 enrichment to be a prerequisite for efficient ex vivo expansion. CD34 selection is an efficient way to debulk cord blood samples from non-clonogenic cells [11, 13]. Those cells might otherwise deplete the culture system from nutritive factors and lead to changes in lactate and pH.

There are some reports about the possible role of accessory cells for the expansion of bone-marrow-derived stem cells, which might also be true for the clonogenic potential of early cord blood progenitors [14]. Since we performed CD34 enrichment with one round of immunomagnetic separation, the purity reached (30%-70%) led to a decrease in

cell concentrations but still left some cells with possible accessory function, e.g., lymphocytes and monocytes in the primary sample. The debulking of the cord blood product is especially desirable when clinical scale expansion is started and the volume of culture media may increase significantly [15]. Nevertheless, recent reports on the existence of $CD34^-$ long-term repopulating cells have to be kept in mind when $CD34^+$ selection is performed [16, 17].

The combination of SCF, FLT-3L, and IL-3 showed the best expansion results. Recently, this combination was described as favorable even for the expansion of SCID repopulating $CD34^-$ stem cells [16].

Like other cytokines, MGDF was found to act not solely on megakaryocytes but also in the expansion of more primitive progenitors, especially when combined with other early-acting cytokines such as Flt3-ligand and SCF [18, 19]. In our experiments, these findings could be confirmed and the increase of $CD34^+/CD41^+$ lineage-determined megakaryocytic progenitors was marked as well.

Taken together, the optimum combination of cytokines in our experiments was SCF, Flt3-ligand, MGDF, and IL-3. Similar growth factor combinations have been shown to conserve the recloning potential of HUCBC in stroma-free cultures for up to 20 weeks [20]. As other authors have shown for bone-marrow-derived progenitors in small [21] and large-scale experiments [22], the addition of EPO, IL-6, and G-CSF tended to improve the expansion results.

Since stroma-derived factors are known to be supportive for the culture of blood stem cells, we tested HUVEC and irradiated BM stroma from healthy volunteers for their influence on the expansion results [23]. As previously speculated, the expansion of total NC, CFU-GM, and $CD34^+$ NC was enhanced by culturing on both feeder layers. Nevertheless, BMSC led to a better expansion than HUVEC. This may be due to the more supportive structure of the BM stroma layer and the different soluble chemokines secreted by both types of feeder cells [24]. BM stroma is known to produce SDF-1, which binds to chemokine receptors such as CXCR-4 on early $CD34^+$ progenitors [25].

To test the influence of stroma contact on the expansion of umbilical cord blood, we performed cultures in dishes where the HUCBC were separated from the feeder layer by microporous membranes. These experiments showed that no-contact cultures which allow only small molecules to traffic between stroma cells and HUCBC improved the expansion results in terms of total NC, $CD34^+$, $CD34^+/38^-$, $CD34/41^+$ cells, and CFU-GM. Other investigators have confirmed that stroma contact might not be necessary for the expansion of LTC-IC [26, 28]. No comparable data are available for HUCBC. Nevertheless, contact might be necessary to preserve in culture the pluripotent, perhaps even $CD34^-$ stem cell. This pre-stem cell might need intermittent cell-cycle arrest in a niche provided by stroma cells to perform asymmetric division and thereby enabling the hematopoiesis to be regenerated for an individual life span. A recent report states the length of the culture to be a crucial factor in terms of loss of primitive pluripotent stem cells [29].

The culture vessels depicted in the **Materials and Methods** section which were rotated during the whole culture period were not able to optimize the expansion results in most cases. Even though the permanent agitation may optimize gas exchange and suspension of the media components [8], no corresponding effects in terms of better culture results were measurable in our experiments.

The rate of apoptosis is known to increase in $CD34^+$ progenitors during in vitro culture and seems to be a useful marker for the assessment of the quality of stem cell grafts [30]. The increased rate of apoptosis may be associated with proliferation and cell division or induced by the cytokines activating more primitive cells [31]. Some authors have found MGDF and other early-acting cytokines to be able to suppress apoptosis in single-cell experiments [32]. The rate of apoptosis measured by annexin positivity in our bulk cultures was not lowered by adding MGDF. Interestingly, there was a trend toward decreased apoptosis in cultures with bone marrow stroma cells, indicating the supportive character of feeder layers.

The addition of stroma-coated microspheres could be a way to ameliorate the results of rotation cultures, but large-scale expansion with these particles in a GMP-like fashion appears to be a difficult task.

The results obtained in our experiments can lead to the design of a new perfusion bioreactor. A compartment containing a feeder layer has to be separated from HUCBC in suspension culture by a semipermeable membrane, allowing the diffusion of soluble chemokines but avoiding direct contact. This so-called "no-contact system" might be perfused with a serum-free medium containing recombinant GMP-grade cytokines. Whether this integrated concept will lead to an expansion of committed progenitors and a conservation of pluripotent stem cells at the same time has to be awaited.

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